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Cytometry Part B (Clinical Cytometry) 94B:23–48 (2018)

Original Article

ICCS/ESCCA Consensus Guidelines to detect GPI-deficient cells in Paroxysmal Nocturnal Hemoglobinuria (PNH) and related Disorders Part 2 – Reagent Selection and Assay Optimization for High-Sensitivity Testing

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Since publication in 2010 of the International Clinical Cytometry Society (ICCS) Consensus Guidelines for detection of Paroxysmal nocturnal hemoglobinuria (PNH) by flow cytometery, a great deal of work has been performed to develop, optimize, and validate a number of high-sensitivity assays to detect PNH phenotypes in both red blood cells (RBCs) and white blood cells (WBCs, neutrophils, and monocytes). This section (Part 2) of the updated ICCS PNH Consensus Guidelines will focus on specific instrument setup for these PNH assays, the identification and proper testing of appropriate antibody conjugates and combinations therof, and basic assay design. © 2017 International Clinical Cytometry Society

Key terms: high sensitivity flow cytometry; paroxysmal nocturnal hemoglobinuria; PNH

How to cite this article: Sutherland DR, Illingworth A, Marinov I, Ortiz F, Andreasen J, Payne D, Wallace PK and Keeney M. ICCS/ESCCA Consensus Guidelines to detect GPI-deficient cells in Paroxysmal Nocturnal Hemoglobinuria (PNH) and related Disorders Part 2 – Reagent Selection and Assay Optimization for High-Sensitivity Testing. Cytometry Part B 2018; 94B: 23–48.

EVOLUTION OF FLOW CYTOMETRIC METHODS TO DETECT PNH

Since the early 1990s, flow cytometric detection of cells lacking expression of glycosylphosphatidylinositol (GPI)-anchored surface molecules has become established as the method of choice for diagnosing and monitoring PNH. Due to the historical association of hemolysis of RBCs with clinical PNH (1–3), early flow cytometric methods to detect PNH relied on detecting the loss of CD55 and CD59, two GPI-linked structures expressed on normal RBCs and WBCs (4,5). Most laboratories used FDA-approved kits (Cellquant and Redquant, BioCytex, Marseille, France) or developed in-house assays that used CD55 and CD59. Typically, RBCs were identified and gated by forward (FS) versus side scatter (SS) in logarithmic mode (FS log vs SS log) and assessed using single-parameter histograms for CD55 and/or CD59 expression. Flow cytometric analysis of RBCs in

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Grant sponsor: Alexion Canada.

Received 19 October 2017; Revised 7 December 2017; Accepted 11 December 2017

Published online 13 December 2017 in Wiley Online Library (wileyonlinelibrary.com).

DOI: 10.1002/cyto.b.21610

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nontransfused patients was used to enumerate the type III (complete GPI-antigen deficiency) and type II (partial GPI-antigen deficiency) PNH clones. Type I (normal GPI-antigen expression) represented remaining normal RBCs. Flow cytometric analysis of WBCs used light scatter in linear mode (FS Lin vs SS Lin) to gate granulocytes in lysed whole-blood samples with similar assessment of CD55 and/or CD59 expression using single-parameter histograms.

ISSUES WITH EARLY FLOW METHODS

Although the ability to rapidly detect GPI-deficient cells by flow cytometry (4–6) has led to improved diagnosis, patient management, and prognosis in PNH and related disorders, simple CD55/CD59-based approaches were neither accurate nor sensitive below the 1%–4% clone size (7), rendering them inadequate to detect the usually small number of PNH phenotypes present in many aplastic anemia (AA) and occasional myelodysplastic syndrome (MDS) cases (8–10). Furthermore, with respect to RBCs, simple "routine" assays that do not employ antibodies to CD235a (anti-glycophorin A) to gate on RBCs are susceptible to the effects of inaccurate pipetting and poor sample mixing (7) and identifying CD55/CD59-negative events that are not RBCs. Such errors can result in false-negative staining with CD55/CD59 antibodies and potentially lead to misdiagnosis.

Inaccurate results ("false positives" and "false negatives") can also be generated by suboptimal instrument setup, selection of poor antibody clones/conjugates that exhibit suboptimal signal-to-noise ratio (S:N), selection of poor conjugates of otherwise good clones, inadequate selection of lineage-specific gating antibodies/conjugates, inadequate reagent titration, contaminated washing media, and poorly designed gating strategies (11).

With respect to the detection of GPI-deficient WBCs, an increasingly wide range of monoclonal antibodies have become available to detect GPI-anchored proteins (12,13), potentially obviating some of the shortcomings of simple CD55/CD59-based assays. Additionally, a variety of more sensitive approaches for PNH detection have more recently been developed utilizing the fluorescent derivative of bacterial pro-aerolysin (FLAER) (11,14–18).

To address these issues, the ICCS published guidelines for the diagnosis and monitoring of PNH and related disorders by flow cytometry (19). A variety of approaches for "routine" and "high-sensitivity" analyses (usually required to detect PNH phenotypes in cases of AA or MDS) were outlined therein for both RBC and WBC lineages. However, standardized-operating protocols (SOPs) utilizing specific reagent cocktails were not identified and defined analytic strategies were not detailed.

THE NEED FOR HIGH-SENSITIVITY FLOW CYTOMETRY FOR THE DETECTION AND MONITORING OF PNH

The development and validation of sensitive, standardized methodologies are essential to reliably detect GPI-deficient PNH phenotypes, especially for PNH clone sizes of <1%. Preliminary data from one large study has shown that 40% of samples that contain GPI-deficient cells do so at a level of 1% or less (see fig. 1, Part 3, Illingworth A, et al., this issue) and a number of the latter will subsequently show an increase in clone sizes (8). Given that standardized, robust, high-sensitivity assays have now been developed (see below), we believe the dichotomy between routine and high-sensitivity assays to be a false one and, from the technical perspective, irrelevant. The same high-sensitivity assays that are required to accurately detect very minor populations of GPI-deficient cells can also be used to detect PNH in patients with large clones; the only difference is in how the assays are deployed with respect to the numbers of cells acquired for accurate and precise determinations of GPI-deficient cell numbers. With the development of standardized methods outlined herein, achieving high levels of sensitivity is not burdensome, provided sufficient cells are collected (see below).

We also believe that both neutrophil and monocyte lineages should be assessed for the presence of GPI-deficient phenotypes as it is not uncommon for monocytes to more accurately reflect PNH clone size than neutrophils (see fig. 12, Part 3, Illingworth A et al. this issue). (Note that we use the more appropriate term "neutrophils" instead of the historical term "granulocytes" throughout this document. Modern gating techniques based on light scatter, CD45 and CD15 (see Parts 2 and 3) result in exclusion of basophils and most eosinophils (although not bands) from the population of interest so that for the most part "granulocyte" clones are in fact "neutrophil" clones. Nevertheless, some figures may still be labeled as granulocytes or "grans.")

INSTRUMENT SETUP: GENERAL CONSIDERATIONS FOR ALL PLATFORMS

The detection of PNH phenotypes by flow cytometry represents a unique challenge in that the assays are designed to detect "negatives," that is, PNH phenotypes have "lost" the antigens of interest, namely, GPI-linked structures. This has considerable implications for how instruments are set up and compensated because the target GPI-negative PNH cell populations (both RBCs and WBCs) need to be visible and fully on scale. The standardized instrument set-up methodologies recommended by the major instrument manufacturers are not necessarily optimal for an assay designed to detect and display PNH phenotypes which requires the antigen-negative population be well resolved from the axis (or origin). This has led many laboratories to establish inappropriate instrument settings to detect these populations (see Appendix A, figures 1–5).

In a more general sense, optimal instrument setup and standardization is a prerequisite for reproducible results over time and among laboratories. Photomultiplier tube (PMT) voltage optimization, standardization, and computer assisted spectral overlap compensation are mandatory steps for instrument standardization on multiparameter assays and can nowadays be performed using an instrument platform-based approach (BD

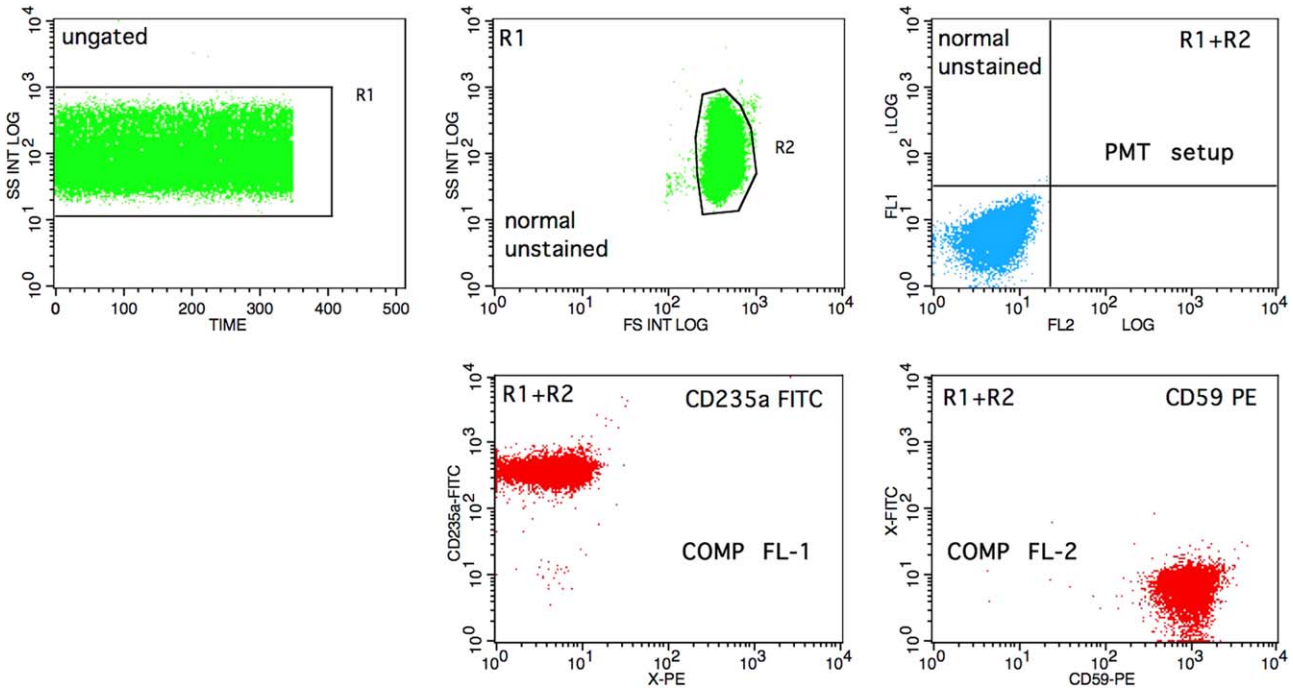

FIG. 1. Recommended instrument PMT and compensation setup for high-sensitivity detection of PNH RBC. Normal unstained RBCs gated by light scatter (log FS/log SS) in R1 (top middle plot) and autofluorescence of unstained control RBCs displayed on PMT 1 versus PMT 2 plot and voltages adjusted until RBCs are well "on scale" (top right). Sample stained individually with CD235a-FITC (bottom left) or CD59-PE (bottom right) and compensation adjustments made to obviate spectral overlap of FITC into PE (bottom left) and of PE into FITC (bottom right). Note: Once light scatter has been optimized, a TIME versus log SS plot (top left) is also established so that fluidics can be monitored during data acquisition. [Color figure can be viewed at wileyonlinelibrary.com]

Biosciences, Beckman Coulter) or inter-platform-based approach (20–23).

Regardless of platform, for the analysis of RBCs, the FS and SS voltages are set in logarithmic mode and voltages adjusted to place the RBC population just beyond the center of the plot and above any forward scatter threshold/discriminator. For WBCs, light scatter voltages are set in linear mode at such values that all leukocyte subsets including lymphocytes scatter above the forward scatter threshold and are clearly clustered on scale.

Regardless of platform, all compensation settings must be set to zero before setting PMTv.

Instrument Setup: Key Points

1. Standard setup instructions from both major manufacturers are optimized for specific applications—and do not transfer directly to PNH testing in either red or white cell assays.
2. FLAER is an Alexa488 conjugate and specific compensation settings are required for this conjugate. Settings optimized for FITC must not be used because of the different spillover of Alexa488 and FITC.
3. Bi-exponential or Logicle plots must be used carefully, as it is important to ensure "negative cell populations," the very targets of testing in PNH (see below) are fully on scale with median channels above the zero axis. In a well-optimized instrument, visualizing the negative population should be similar when viewed in either logarithmic or bi-exponential/logicle mode.
4. The appropriate target population must be used for PMTv settings. For the red cell assay, unstained red blood cells are appropriate; for the white cell assay, it is important to ensure the unstained lymphocyte population is clearly on scale.

RECOMMENDED INSTRUMENTATION SETUP AND COMPENSATION TO DETECT GPI-DEFICIENT PNH CELLS: ALL INSTRUMENTS

Given the issues noted in Appendix A, figures 1–5, with respect to generic instrument setup and compensation, what follows describes the recommended best practices for PNH assay-specific instrument optimization.

RED BLOOD CELLS

Sample and Reagent Requirements

Whole anticoagulated (EDTA is preferred/most tested but Heparin or ACD are also acceptable) peripheral blood is diluted 1:100 with clean phosphate buffered saline (PBS). CD235a (glycophorin A), is expressed in high copy number on mature RBCs and CD235a antibodies represent the only means to fully delineate or "gate" RBCs from other cellular and noncellular events (11,19). To detect GPI-deficient PNH RBC phenotypes, antibodies to CD59 provide the best means to delineate and enumerate PNH type III, PNH type II and normal

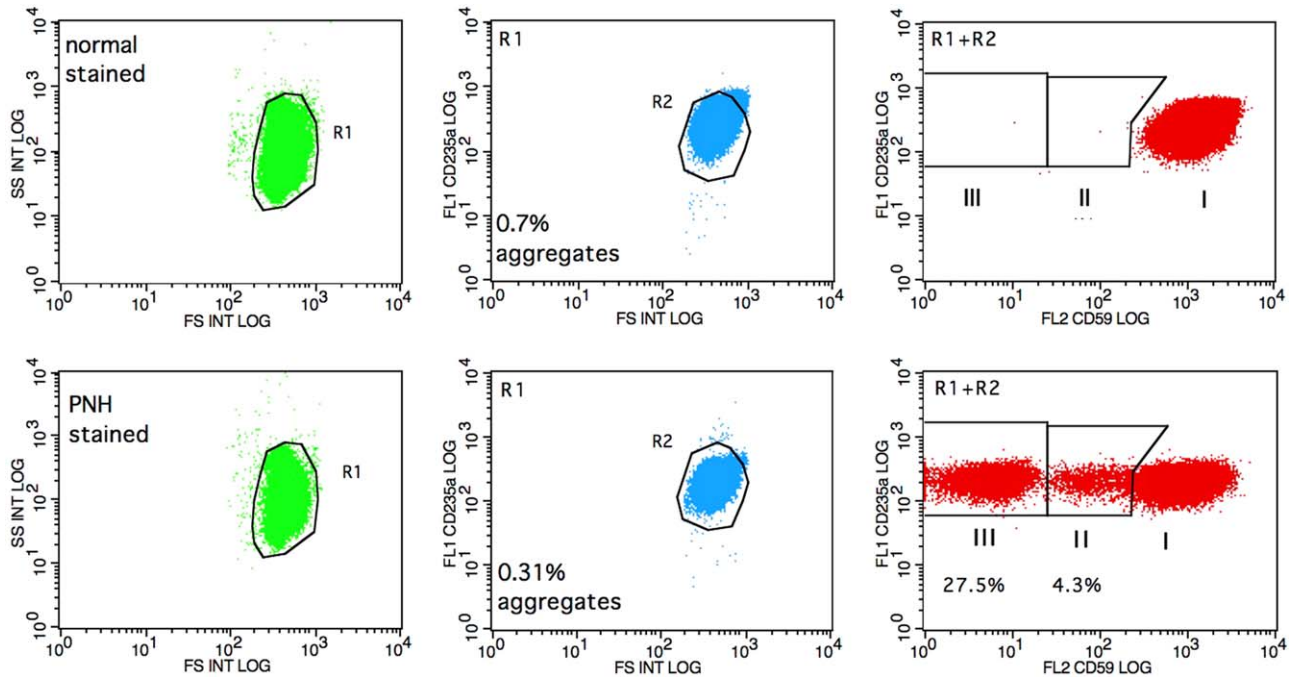

FIG. 2. Stained normal and stained PNH sample. Top row: Normal RBCs were stained with a cocktail comprising CD235a-FITC and CD59-PE, and washed/racked sample acquired using the instrument settings established in Figure 1. RBCs were sequentially gated through TIME versus log SS, and log FS versus log SS (top left) as shown in Figure 1 and displayed on CD235a-FITC versus log FS plot (top middle). CD235a+ RBCs were gated in R2 and aggregated RBCs (0.7% of total) excluded as shown. Cells from the TIME plot and R1 and R2 were displayed on CD235a-FITC versus CD59-PE (right). A second sample stained only with CD235a-FITC was analyzed to approximate the location of the Type III gate (see Figure 6 bottom left). The upper range of the Type II gate is drawn proximal to the stained normal RBCs. Bottom row: A PNH sample was stained and analyzed as described for the normal sample. Aggregated RBCs (0.31%) were excluded by gate R2. The sample contained 27.5% PNH Type III cells and 4.3% PNH Type II cells. [Color figure can be viewed at wileyonlinelibrary.com]

(type I) RBCs (11,19). Once optimal conjugates of CD235a and CD59 have been identified and titrated (see below), it is important to make a diluted "cocktail" of these reagents to avoid the generation of staining artefacts caused by pipetting and/or sample-antibody mixing errors. When individual pipetting of very small volumes of individual antibodies is performed, failure to add or admix the CD59 reagent properly can generate "false" PNH phenotypes (7,11). When a premixed cocktail is used, staining artefacts can be readily identifed on two-dimensional plots by their lack of proper staining with both CD235a and CD59 (11 and figure 6 of Supplementary Data).

Instrument Set-Up and Compensation

The "TIME" parameter must be collected so that fluidics can be monitored during data acquisition (Fig. 1, top left) and if necessary, artifacts generated by the introduction of air bubbles can be avoided and/or excluded during analysis (see Part 3, Illingworth A et al., this issue).

Light scatter voltages must be set in logarithmic mode for both FS and SS and voltages adjusted to place the cluster of RBCs just beyond the middle of the plot obviating the possibility that some red cells may fall below the forward scatter discriminator/threshold. At this setting, if any aggregates of RBCs are present, they will be visible; aggregates show up as a second population exhibiting slightly higher forward and side scatter characteristics (Fig. 1, top middle). Regardless of instrument used or voltage levels used, care must be taken to ensure that the forward scatter threshold/discriminator does not exclude any *bona fide* singlet RBCs.

With compensation turned off or "zeroed," PMTv for FL1 and FL2 should be set with unstained red blood cells, ensuring that the cells are comfortably "on-scale" in both PMTs (Fig. 1, top right). This step is performed in exactly the same manner regardless of flow cytometry platform in use. For instruments with bi-exponential/logicle display capabilities, such should be turned "off" when establishing PMTv.

Compensation is set by removing fluorescence spillover into the PE channel with an RBC sample stained only with CD235a–FITC (Fig. 1, bottom middle). Similarly, spillover of PE into the FITC channel is removed using a sample stained only with CD59-PE (Fig. 1, bottom right). Adequate compensation is verified using a normal sample stained with "cocktailed" CD235a–FITC and CD59–PE (Fig. 2, top) and a PNH sample (Fig. 2, bottom). The type III gate is established using a normal sample stained only with CD235a–FITC, and the upper limit of the type II gate is established using a normal sample stained with cocktailed CD235a–FITC and CD59–PE.

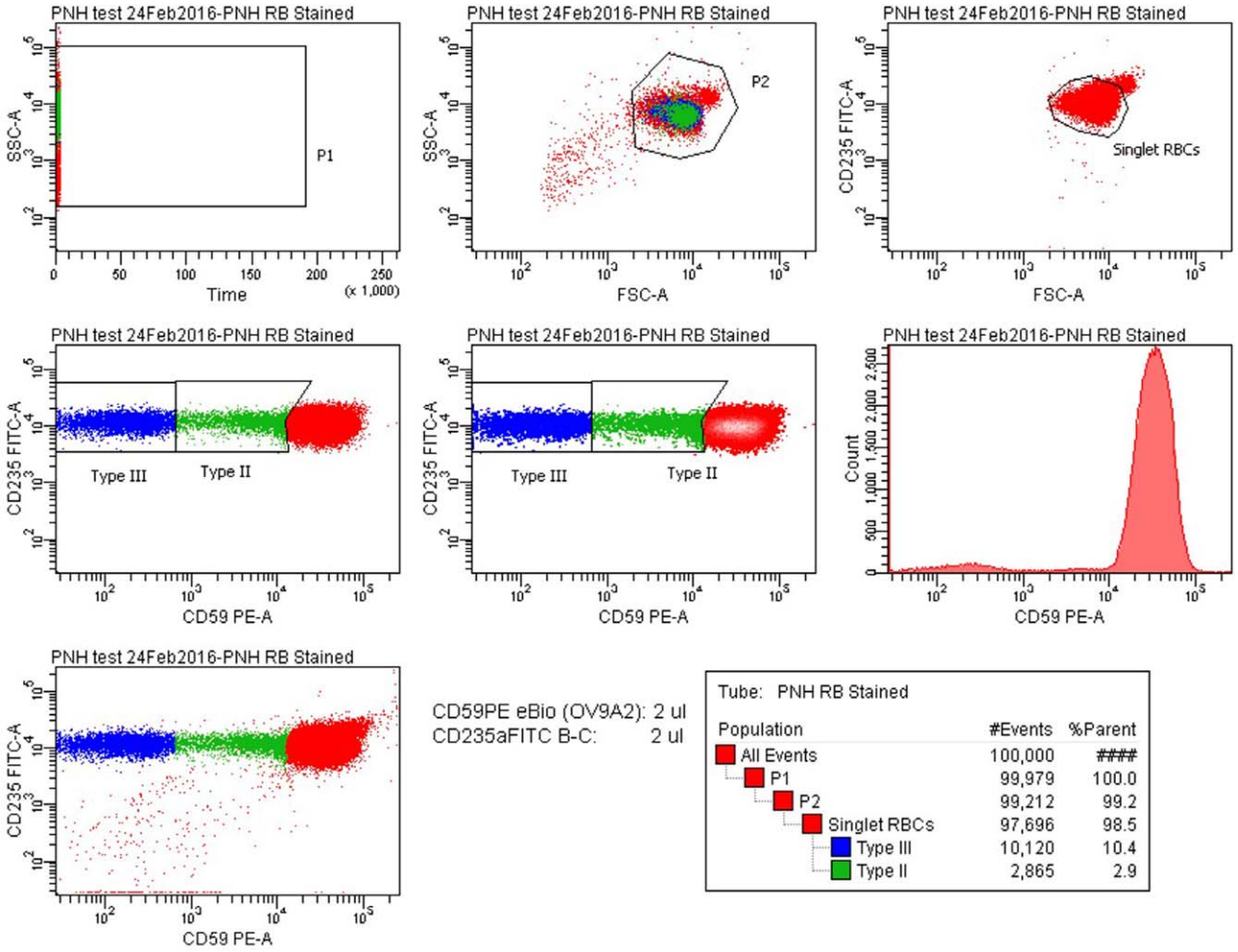

FIG. 3. Analysis of PNH sample on an FACSCanto II cytometer showing all Boolean gating steps. Sample stained as Figure 2 and instrument PMT and compensation adjustments performed exactly as described. The relative sizes of the PNH Type III and Type II populations can be derived from the dot plot (middle left) or the density plot (middle row middle). The Type III and Type II gates are linked across these two plots. The single parameter histogram (middle row right) is not always as informative as either dot plot or density plot but is useful in comparing reagent cocktail lots.

Essential details of antibody cocktailing, RBC staining, washing (twice) and racking procedures are presented in ref. 11, plus Supplementary Data and refs. 23–27.

The data presented in Figures 1 and 2 were acquired on FC500 and Navios instruments. The FCS2.0 component was exported using Flowjo software (Treestar Inc.) and analyzed in Cellquest Pro (BD Biosciences), while that shown in Figure 3 was acquired on a 3-laser Canto II cytometer set up exactly as described (11,25–27).

REAGENT SELECTION: RED BLOOD CELL ASSAY

CD235a Gating Antibodies for RBCs

CD235a is a heavily O-glycosylated negatively charged sialo-glycoprotein. A screen of a number of CD235a–PE conjugates demonstrated many of those tested caused unacceptable levels of RBC aggregation even after extensive titration. However, the use of negatively charged FITC conjugates minimized the aggregation caused by PE-conjugated forms of the very same clone while maintaining a decent specific staining signal (7,11,25–27). For such reasons, a number of FITC conjugates of different clones were investigated in more detail and to date clones YTH 89.1 (Cedarlane), 10F7MN (eBioscience/Thermo Fisher), JC159 (Dako), and KC16 (Beckman Coulter) have been tested and validated (Table 1). FITC conjugates of other CD235a clones have also been tested but shown to suboptimal (11 and Supplementary Data).

Importance of CD235a–FITC Conjugate Titration

In addition to using visual assessment of the individual plots to evaluate the optimal titration of an antibody conjugate, several semi-quantitative methods have been developed. The simplest of these, the signal-to-noise ratio (S:N) measures the median channel fluorescence intensity (MFI) of the stained population and compares this with the MFI of the unstained "internal control" or

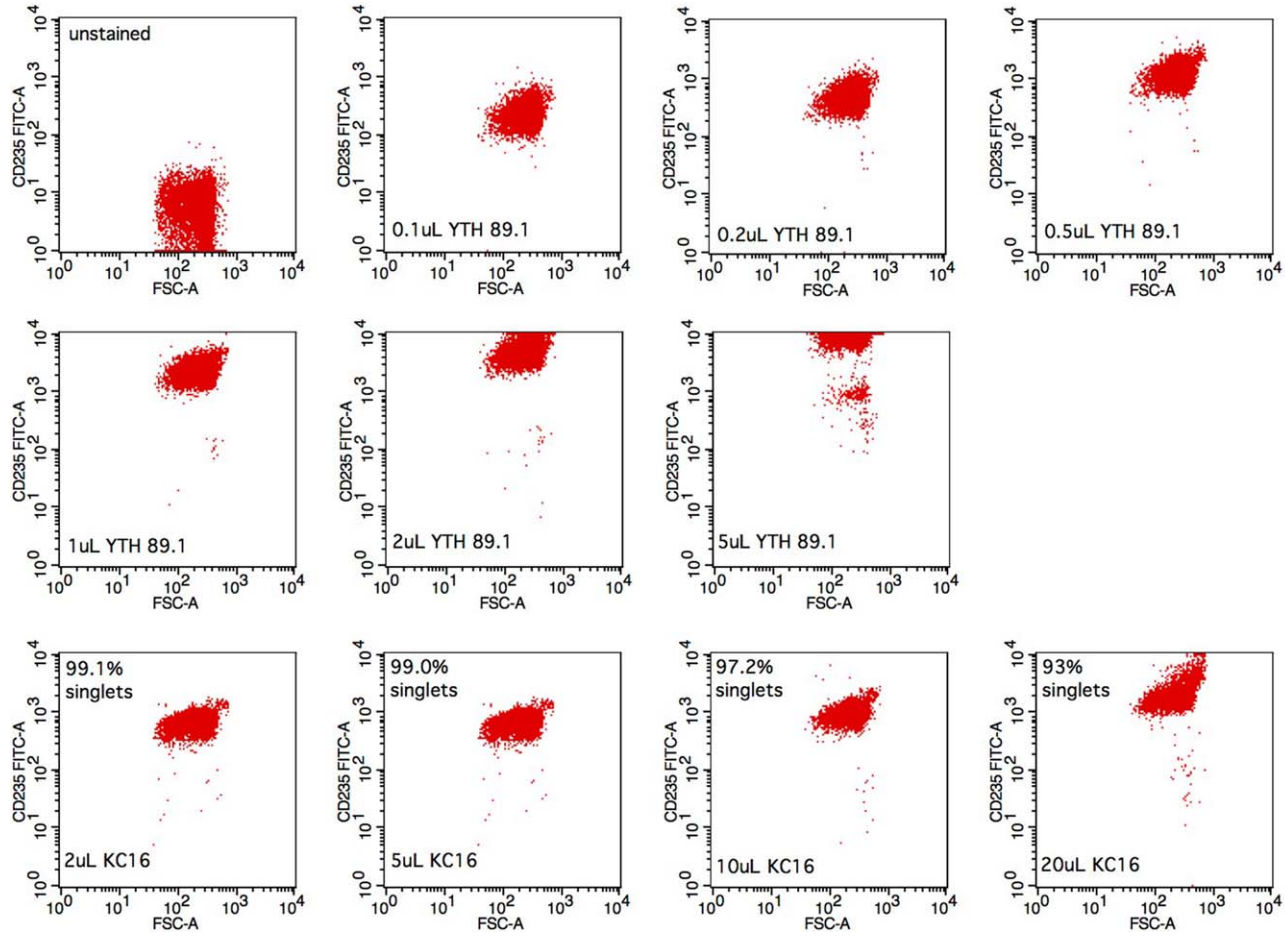

FIG. 4. Titration of two CD235a–FITC conjugates on a normal PB sample. Effects of increasing reagent concentration on staining intensity and RBC aggregation. Top two rows show YTH 89.1 titration and bottom row shows KC16 titration. [Color figure can be viewed at wileyonlinelibrary.com]

negative population. The Stain Index (SI) is more sophisticated and also takes into account the spread of the negative population. Titration of CD235a conjugates to derive S:N or SI data are rendered impossible however by the fact that all RBCs express CD235a, thus there are no internal unstained or negative populations available to properly perform S:N or SI calculations.

Furthermore, the amount of aggregation varies from clone to clone and also varies depending on the conjugated form of any specific clone. Such effects cannot be detected by looking at S:N and SI values.

Thus, to determine the optimal volume of any specific CD235a conjugate to use, one needs to look at the flow data (dot plots) of the titration. As can be seen in Figure 4 (bottom row), the staining intensity of the RBCs is entirely adequate at 2 μL KC16/test for this particular lot, with only minimal evidence of aggregated RBCs. While there is a slight increase in the staining intensity at increasing volumes, there is a concomitant increase in the amount of RBC aggregation. As additional aggregation can be anticipated when CD59 is added to this assay, it is important to take RBC aggregation into account when optimizing the volumes of CD235a–FITC to be used.

When the flow plots of the YTH 89.1FITC titration are observed (Fig. 4, top two rows), it is clear that the optimal volume is in the 0.25 μL range for this conjugate.

Finally, it has been noted that some CD235a–FITC conjugates do not stably stain RBCs and once washed, staining intensity can drop significantly over time. To minimize this issue, regularly noted with every lot of KC16-FITC tested to date, it was previously recommended that RBCs stained with this reagent need to be acquired no >15 min post washing and racking (11,25,26). To investigate this further, RBCs were stained with 10, 5, 2.5, or 1.25 μL of either KC16-FITC, 10F7MN–FITC, or with 1, 0.5, 0.25, or 0.125 μL of YTH 89.1–FITC. Samples were washed twice, racked, and acquired as soon as possible with the KC16–FITC stained samples first, then 10F7MN–FITC stained samples and finally those stained with YTH 89.1. After initial data collection, all samples were left in the dark at room temperature for 75 min and all samples were reacquired. As shown in Figure 5, an average of about 65% of the KC16–FITC staining was lost after 75 min, with similar data being observed across the titration range. In contrast, only about 16% of the staining intensity of the

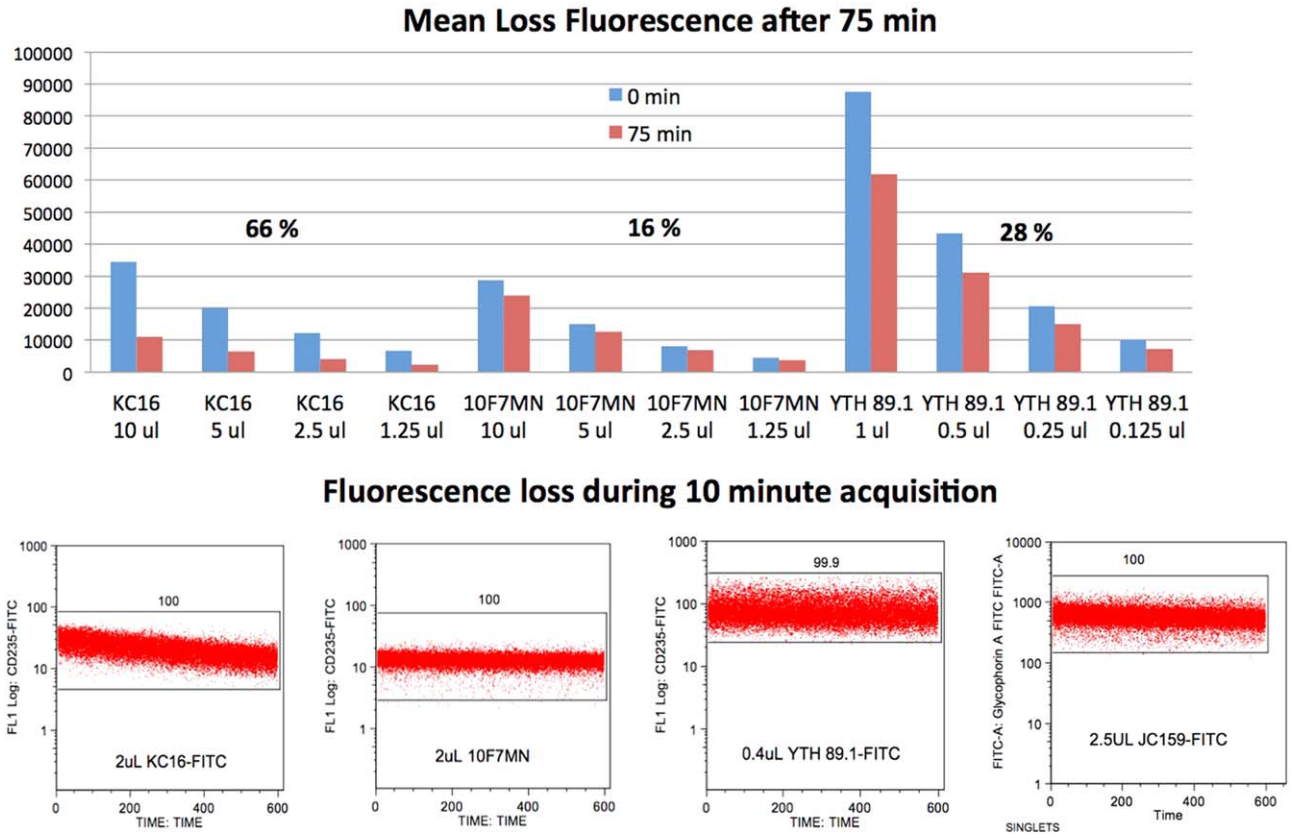

FIG. 5. Comparison of CD235a–FITC conjugates. Effect of time on staining intensities. Top: Titrations were performed with clones KC16, 10F7MN, YTH 89.1 and data acquired immediately after washing/racking. Left-over stained samples were kept at room temperature in the dark for 75 min and then data reacquired. After 75 min, there was a mean loss of fluorescence of 66%, 16%, and 28% for KC16, 10F7MN, and YTH 89.1, respectively. Bottom: Normal PB sample stained with clones KC16, 10F7MN, YTH 89.1, or JC159 and data acquired for 10 min to monitor mean fluorescence loss over this time period. [Color figure can be viewed at wileyonlinelibrary.com]

10F7MN–FITC and about 28% of the YTH 89.1–FITC conjugates, respectively, were was lost over the same period. To better assess the implications of this data, we stained a sample with optimized volumes of each conjugate. After washing and racking, we performed a 10 min data acquisition on each stained sample. As can be seen from the TIME versus CD235 staining plots (Fig. 5), there is a significant decrease of staining observed for the KC16–FITC conjugate after only 10 min, whereas the 10F7MN–FITC, YTH 89.1–FITC, and JC159–FITC conjugates showed no major decrease of fluorescence over this time period. This is significant because long data acquisition times (several minutes) can be required on some instruments for this assay to collect enough cells for precise measurements in samples with small numbers of PNH RBC phenotypes.

Antibodies to GPI-Linked Structures on RBCs

Historically, CD55 and CD59 were used to detect the presence of GPI-negative PNH phenotypes in the RBC lineage. However, extensive assessment of CD55 and CD59 clones/conjugates has indicated that properly validated conjugates of CD59 best delineate type III and type II PNH phenotypes from normal (type I) RBCs 11, plus Supplementary Data, 19,24–26 and that dim staining with CD55 conjugates can be misleading. Similarly, the use of CD58, another GPI-linked structure expressed on RBCs, does not improve the fidelity of the CD59-based PNH RBC assay (DRS unpublished). Phycoerythrin (PE) is the fluorochrome of choice for the CD59 conjugate as it is the brightest nontandem fluorochrome available for clinical instruments equipped with an argon 488 laser.

Importance of CD59–PE Conjugate Titration

As for the CD235a–FITC gating reagent, the CD59–PE conjugate must be extensively titrated to maximize staining signal while minimizing the aggregation of RBCs caused when working close to saturation levels of CD59–PE conjugates. Similarly to CD235a, CD59 stains all normal RBCs, so that there are no internal negative controls in such samples against which to properly measure S:N and SI. Alternatively, PNH samples that contain *bona fide* PNH type III populations that can be unequivocally delineated from PNH type II cells can be used to assess and titrate CD59 conjugates using both visual assessment of plots and by using the S:N and SI

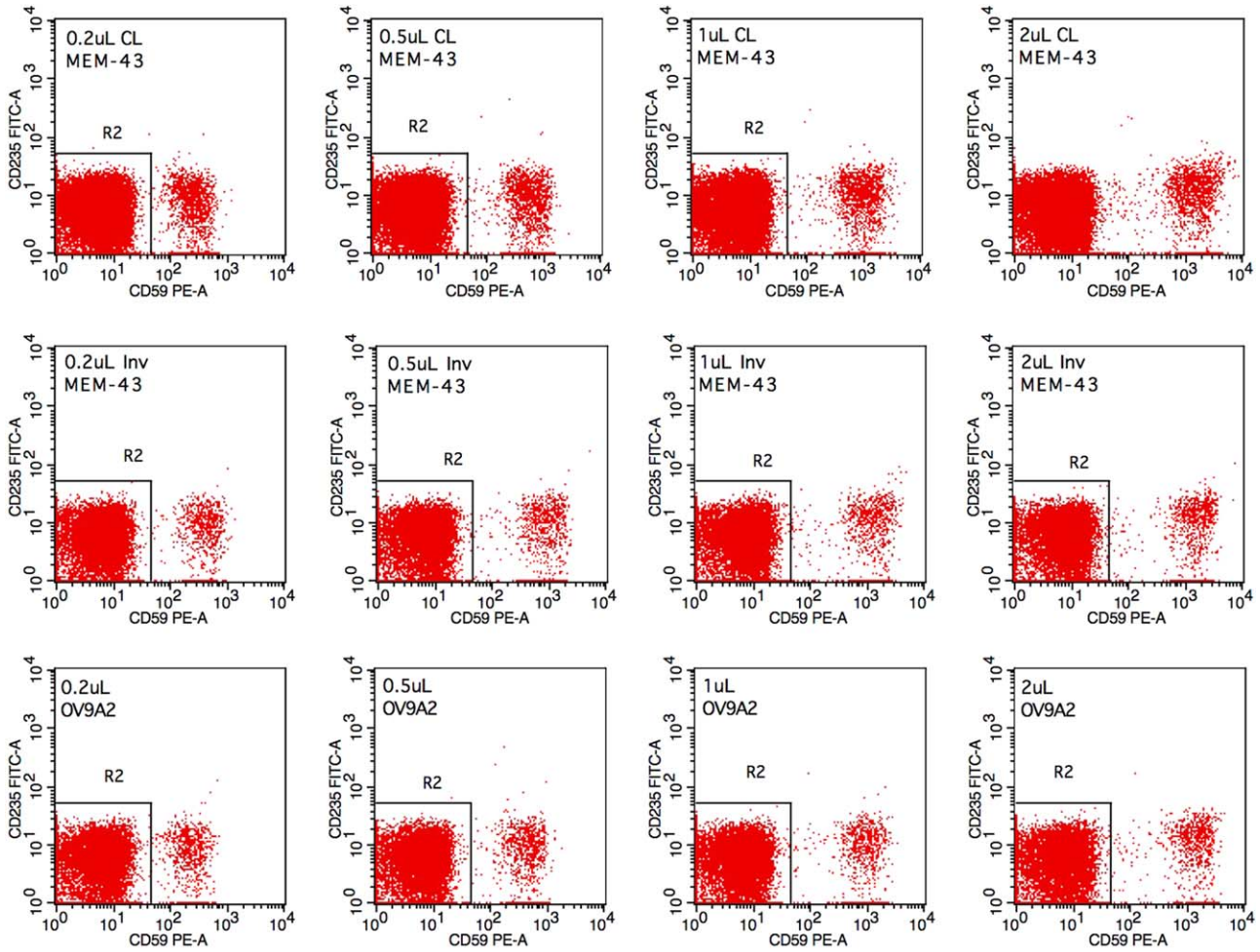

FIG. 6. Titration of recommended CD59PE conjugates. Top; clone MEM-43 sourced from EXBIO/Cedarlane. Middle; MEM-43 sourced from Invitrogen/Thermo Fisher. Bottom; OV9A2 sourced from eBio/Thermo Fisher. [Color figure can be viewed at wileyonlinelibrary.com]

calculations. Outside of major testing centers, such samples may not be regularly encountered.

We have used both approaches to screen a number of CD59 clones in a variety of conjugated forms, focusing in particular on PE-conjugated versions, and we have identified several reagents that perform well (PE-conjugates of clones MEM-43 and OV9A2).

Table 1
Recommended CD235a–FITC and CD59–PE Conjugates for High-Sensitivity PNH RBC Assay

Target	Antibody conjugates	Purpose	Clone and vendor
RBC	CD235a–FITC	Gating on RBC	10F7MN (eBioscience) YTH 89.1 (Cedarlane) KC16 (BC)[a] JC159 (DAKO)
	CD59–PE	GPI-linked for RBC	OV9A2 (eBio) MEM-43 (Invitrogen) MEM-43 (EXBIO/Cedarlane)

[a]RBCs stained with this conjugate must be acquired immediately as staining diminishes within 10 min.

Figure 6 shows the titration of two different PE conjugates of MEM-43 sourced from Cedarlane (top row) and Invitrogen/ThermoFisher (middle) alongside the PE-conjugated version of OV9A2 (obtained from eBioscience prior to the latter being acquired by Thermo Fisher Inc.) (bottom) on a PNH sample containing about 95% PNH type III RBCs and virtually no PNH type II cells. These plots suggest 1 μL of each reagent would be a close to optimal volume to use in each case. Above 2 μL of these reagents, increased background staining of PNH type III cells and increased levels of aggregation were noted (data not shown).

We have also identified PE conjugates of other clones that are totally inadequate for purpose (PE conjugates of clones P282 and 1F5). As shown in Figure 7 the same PNH sample with the 95% type III PNH clone was also used to test the 1F5 CD59–PE conjugate (middle row) in combination with 0.25 μL of YTH 89.1 CD235a–FITC. For comparison, the same sample stained with the YTH

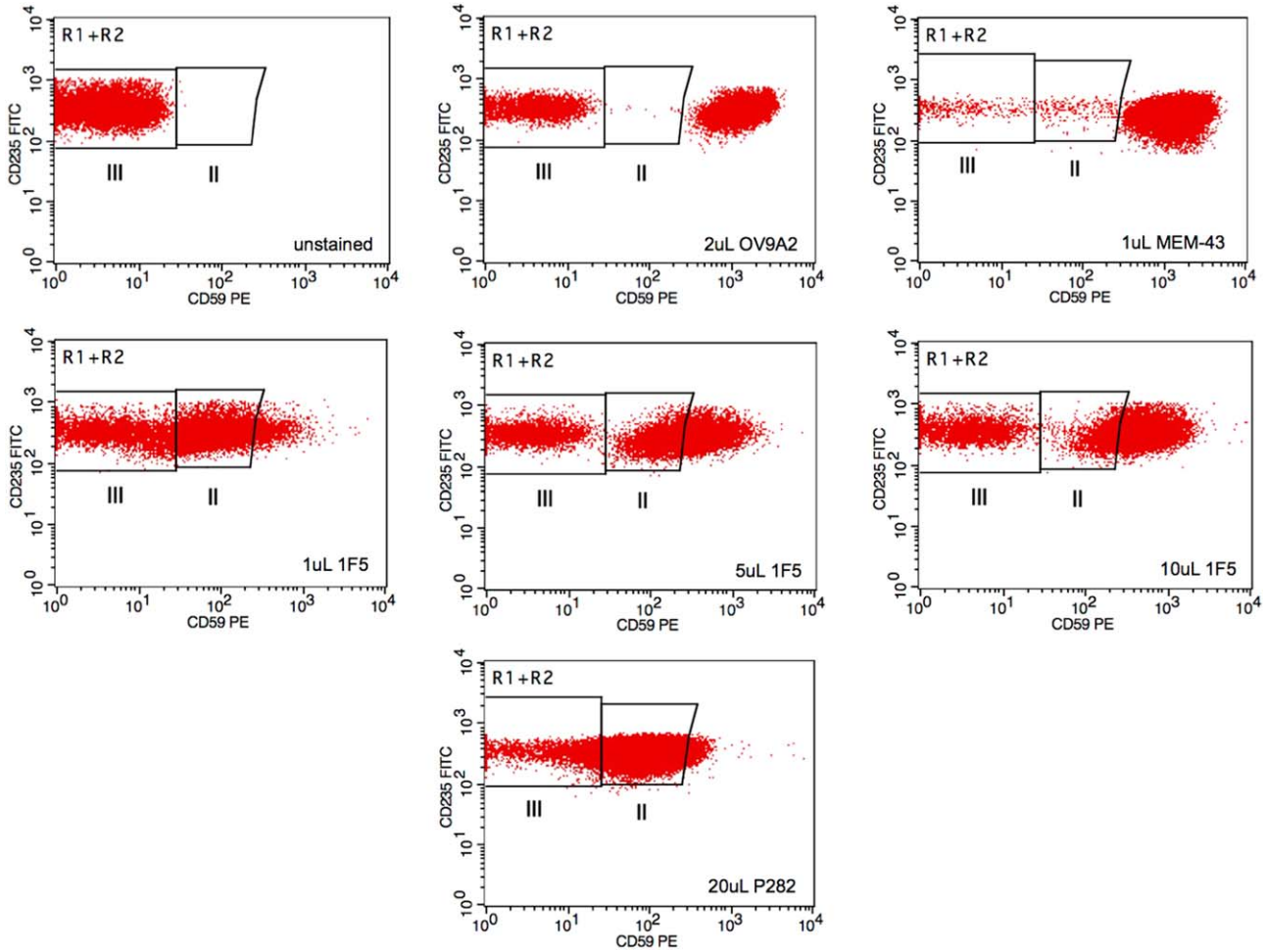

FIG. 7. Comparison of recommended and nonrecommended CD59–PE conjugates. Top left; Unstained cells from a PNH sample. Top middle; 2 μL of OV9A2 on PNH sample. Middle row, titration of clone 1F5-PE on same PNH sample; left to right 1, 5, and 10 μL IF5-PE. Top right, 1 μL MEM-43-PE on AA sample with small Type III and Type II RBC populations. Bottom middle, same AA sample stained with 20 μL of clone P282- PE. [Color figure can be viewed at wileyonlinelibrary.com]

89.1 and 2 μL OV9A2–PE conjugate is shown in the top middle plot. The top right plot shows an AA sample containing small PNH type III and PNH type II RBC populations stained with a combination of 2 μL KC16 CD235a–FITC and 1 μL of MEM-43 CD59–PE. The same AA sample stained with KC16 CD235a–FITC and 2 μL of P282 CD59–PE is shown on the bottom plot. With respect to the P282–PE conjugate at least, this is not a "clone" issue, but a conjugate issue; this clone performs entirely adequately in FITC-conjugated form (11 and Supplementary Data).

In summary, when assessing, validating (cellular reactivity), and titrating an antibody conjugate for use in the PNH RBC assay, one has to look at the data plots to get the full picture. Not many laboratories will have access to PNH cases specifically containing the clearly delineated PNH type III RBC populations needed to perform proper S:N, and SI calculations. Even for those that do, the graphs of such analysis are not informative when the cells are stained beyond the fluorescence limits of the plots and such analysis fails to address the significant RBC aggregation that occurs well before antibody saturation is reached. Such analysis also fails to identify other issues such as the significant loss of CD235a–FITC fluorescence over time of some otherwise validated CD235a–FITC conjugates.

Sample Staining and Data Acquisition

A fresh anticoagulated blood sample (<48 h old) is diluted 1:100 with clean phosphate buffered saline. Using reverse-pipetting to avoid aerosols, 100 μL of the diluted PB is pipetted directly into the bottom of the staining tube and the tip carefully removed to avoid leaving any sample on the sides of the tube. A diluted cocktail of CD235a–FITC/CD59–PE is made. In a typical example, 20 μL of the KC16 CD235a–FITC conjugate is mixed with 5 μL of the MEM-43 CD59–PE conjugate and brought to 200 μL by addition of clean PBS. 20 μL of the diluted cocktail is added with regular pipetting technique directly into the blood sample in the staining tube

and mixed with gentle up-and-down pipetting. The pipette tip is then carefully removed to avoid leaving any sample on the side of the tube. The tube can then be gently "swirled" with a vortex set to very low speed, taking care to avoid aerosols. After incubation for at least 20 min in the dark at room temperature (incubation for up to 60 min has been validated by the authors), the stained cells must be washed twice with clean PBS, before being resuspended in 1 mL of PBS. Finally, just before acquisition, the sample is "racked" vigorously by dragging the staining tube 3–4 times across a hard plastic or metal test tube rack. This last step is critical to the disruption of RBC aggregates generated by the staining process.

While Figure 3 shows an acquisition/analysis template from a Canto II cytometer, essentially identical templates and Boolean gating strategies are used on FACSCalibur, FC500, and Navios platforms (see Part 3 Illingworth A et al., this issue). In general, at least 100,000 gated CD235a+ RBCs are collected. If any PNH type III events are detected, up to 1 million RBCs may need to be collected (11,19,24–26).

Detailed validation of RBC assay is described in Part 4 (Oldaker T et al., this issue).

WHITE BLOOD CELLS

Sample and Panel Requirements

Undiluted anticoagulated whole blood is the preferred sample source for the analysis of PNH phenotypes in WBCs. The inclusion of a pan–CD45 conjugate in the staining cocktail is highly recommended. Along with a light scatter plot, CD45 is highly effective at removing subcellular debris, platelets and unlysed RBCs and stains peripheral blood samples in a highly predictable manner allowing for "pattern recognition." Furthermore, it is not uncommon for previously undiagnosed leukemia samples to be submitted for PNH testing and the presence of CD45 in the staining cocktail is highly advantageous as leukemic blast cells form discrete CD45+ (dim) clusters on CD45 versus side scatter plots (16).

As outlined previously (11,19), two GPI-linked structures must be analyzed per WBC lineage assessed. Combinations of FLAER and CD24 or FLAER and CD157 represent the most tested combination of reagents to detect GPI-deficient neutrophils, while FLAER in combination with either CD14 or CD157 represent the most validated combination of reagents to detect GPI-deficient monocytes (11,25–27).

However, the development of high-sensitivity assays owes less to the availability of properly validated and superior GPI-specific conjugates available nowadays than it does to the use of good lineage-specific "gating" reagents. In the past, CD33 was widely used to "gate" both neutrophils and monocytes (7,16). However, there are several drawbacks to this approach. On "difficult" sample types, for example, MDS, CD33 is usually inferior to CD15 for gating neutrophils. Furthermore, CD33 fails to delineate platelet aggregates when present, from mature neutrophils and such "contaminants" can mimic PNH neutrophils on FLAER versus CD24 plots. Finally, CD33 expression in some rare cases can be very low or negative (11,25–27).

With respect to monocytes, CD33 is not effective at delineating monocytes from basophils and the latter can mimic PNH monocytes due to their low binding of FLAER and lack of staining with CD14. In contrast, CD64 is highly expressed on monocytes but is not expressed on basophils. Myeloid blasts if present can mimic monocytes in their expression of CD33 and such cells are difficult to delineate from PNH monocytes (especially if CD45 is not also used in the gating strategy) as they express very low levels of FLAER and are usually negative for CD14. On some MDS cases, CD64 also outperforms CD33 as a monocyte-gating reagent, and as indicated above, we have encountered occasional samples that do not express CD33. The sensitivity of PNH assays that depended on the use of CD33 for gating neutrophils and monocytes was thus limited to around 1% due to the high background rate of PNH phenotypes in some sample types (7,28).

The use of CD15 for gating neutrophils and CD64 for gating monocytes obviates many of these problems (11,24–27) on the vast majority of samples, even though there are occasional MDS cases in which it can still be very difficult to delineate the target neutrophils and monocytes. However, CD64 may be upregulated in activated neutrophils that may make the separation between monocytes and neutrophils challenging in such cases.

Instrument Set-up and Compensation: White Blood Cells

To analyze white blood cells, stained samples must be subject to an RBC lysis step. A variety of commercial RBC lysing agents are available for this, most of which include fixatives. While ammonium chloride-based lysing agents can also be used, neutrophil degranulation continues in the absence of fixatives, rendering analysis more challenging and thus NH4Cl-based lysing is not generally recommended unless a subsequent fixative step is included in the procedure. Regardless of lysing agent used, the cells need to be washed thereafter with phosphate-buffered saline supplemented with 1% bovine serum albumin before acquisition.

For instruments that do not automatically save "TIME" as a parameter, this parameter must be collected so that fluidics can be monitored if needed.

A multistep Boolean gating approach has been adapted to efficiently gate neutrophils and monocytes that begin with setting light scatter voltages in linear mode to ensure that all cells of interest (neutrophils and monocytes for PNH "clone" detection and lymphocytes used as internal staining controls) are properly on-scale and above the threshold/discriminator, used to remove subcellular debris.

As per the RBC assay, compensation must be zeroed when establishing PMTv.

Setting the PMTv

While bead-based approaches can be used to establish the PMTv, the authors strongly recommend the use of cells, especially for this assay. Although unstained lymphocytes gated from a light scatter plot can be used for this, a more sophisticated and recommended approach involves using the unstained subset of lymphocytes in a sample that has been stained with a lymphocyte subset-specific antibody. For example in a sample stained with a CD3 conjugate, the stained, lysed and washed sample is acquired and the PMTv established to ensure that unstained B-lymphocytes in the sample are comfortably on scale and not crushed on the axis. For instruments with logicle or bi-exponential displays, such should be turned off during PMTv setting. To set other PMTv to be used in the assay, a similar approach can be employed using CD3 (or similar antigen with distinctly separated unstained and stained populations) conjugated to the appropriate fluorochromes specific for the fluorescence channels to be used in the assay. When FLAER-Alexa488 will be used as recommended in the final assay, it is acceptable to use a CD3–FITC conjugate to establish the FL1 PMTv. However, it must be noted that specific compensation requirements for Alexa488 will need to be established (see below).

PMTv standardization

For PMTv optimization, we define fluorescence target values (MFIv) for all fluorochromes used in the assay. A mandatory step for instrument standardization and reproducible data in time and between instruments is the maintenance of the target MFIv by adjusting PMTv. For BD platform PMTv standardization could be performed automatically using the CS&T performance check and application settings, while for BC platform and the instrument platform independent EuroFlow and Harmonemia approaches, PMTv standardization should be performed manually using the Flow-Set Pro fluorospheres and 8-peak Rainbow beads, respectively (20–23).

Establishing the compensation matrix

Cells. While spillover from specific fluorochromes into adjacent PMTs can be corrected by compensation using cells individually stained with saturating volumes of CD45 conjugates and the auto-scheduler (on Beckman Coulter instruments), the use of this methodology has often resulted in overcompensation, causing target populations of interest, namely PNH phenotypes, to be "crushed" on the axis (Appendix A, Figure 5). A more recently described, cell-based option that has utility on both platforms involves the use of optimally titrated CD3 conjugates (or conjugates of other antibodies that clearly delineate subsets of lymphocytes, e.g., CD8, CD19) for the compensation process (see: http://www.cytometry.org/web/modules/module2.pdf for Beckman Coulter instruments and http://www.cytometry.org/web/modules/module3.pdf for BD Biosciences instruments).

Note: For assays that include FLAER-Alexa488, a sample must be stained with FLAER, and gated FLAER+ lymphocytes are used to generate the compensation matrix. Finally, after the auto-scheduler establishes the compensation matrix, it is verified with a sample stained with the full reagent cocktail used for the PNH assay and any further optimization of the matrix can be performed.

Antibody capture beads. Alternatively, antibody capture beads can be used to generate the compensation matrix. Again, there are a variety of commercial products available to do this and all of those tested to date appear to work well and across instrument platforms. *Note*: for setting compensation with any antibody capture bead-based method, the use of an Alexa488 conjugate of CD3 (or similar) is required. Individual aliquots of antibody capture beads are singly stained with each antibody in the WBC staining cocktail to be used. For example, (as shown in fig. 15 of ref. 26) in a FLAER-based 6-color assay on a 10-color Navios cytometer, individual aliquots of VersaComp™ capture beads can be stained with CD3-Alexa488 (eBioscience/Thermo Fisher), CD24-PE, CD15-PC5, CD64-PC7, CD14-APCA700, and CD45-KO (all Beckman Coulter). Unstained beads are added to each stained aliquot to provide unstained beads in each stained sample, and the matrix is generated in software using the fluorescence of the stained and unstained beads.

A very similar approach can be used on Canto cytometers as the bead-based compensation software in FACS-Diva has an option to include unstained beads in the same tube as the stained beads. Alternatively, unstained beads are acquired before the individually stained bead samples and the compensation matrix is then generated in software.

Regardless of methodology used to establish the compensation matrix, some manual optimization will likely be required using a sample stained with the reagent cocktail in use.

REAGENT SELECTION: WHITE BLOOD CELL ASSAYS

The ICCS Guidelines featured a Table listing some possible reagent combinations potentially suitable for a variety of instrument platforms with 3-, 4-, 5-, or 6-PMTs (see Appendix A in ref. 19). Specific antibody clones and specific conjugates thereof however were not identified at that time. Since then a large number of specific clones/conjugates have been assessed for lineage-specific gating and detection of GPI-deficient neutrophils and monocytes. Optimal titration ranges were established and selected conjugates validated both individually and in combination with other reagents on both normal and PNH samples. Using this information, high-sensitivity assays were developed using 4-color cocktails based on FLAER, CD24, CD15, and CD45 (for neutrophils) and FLAER, CD14, CD64, and CD45 (for monocytes) (11, plus Supplementary Data). These robust assays were developed for all clinical cytometers with 4 or more PMTs and were capable of successful deployment beyond the original developers' laboratories (29).

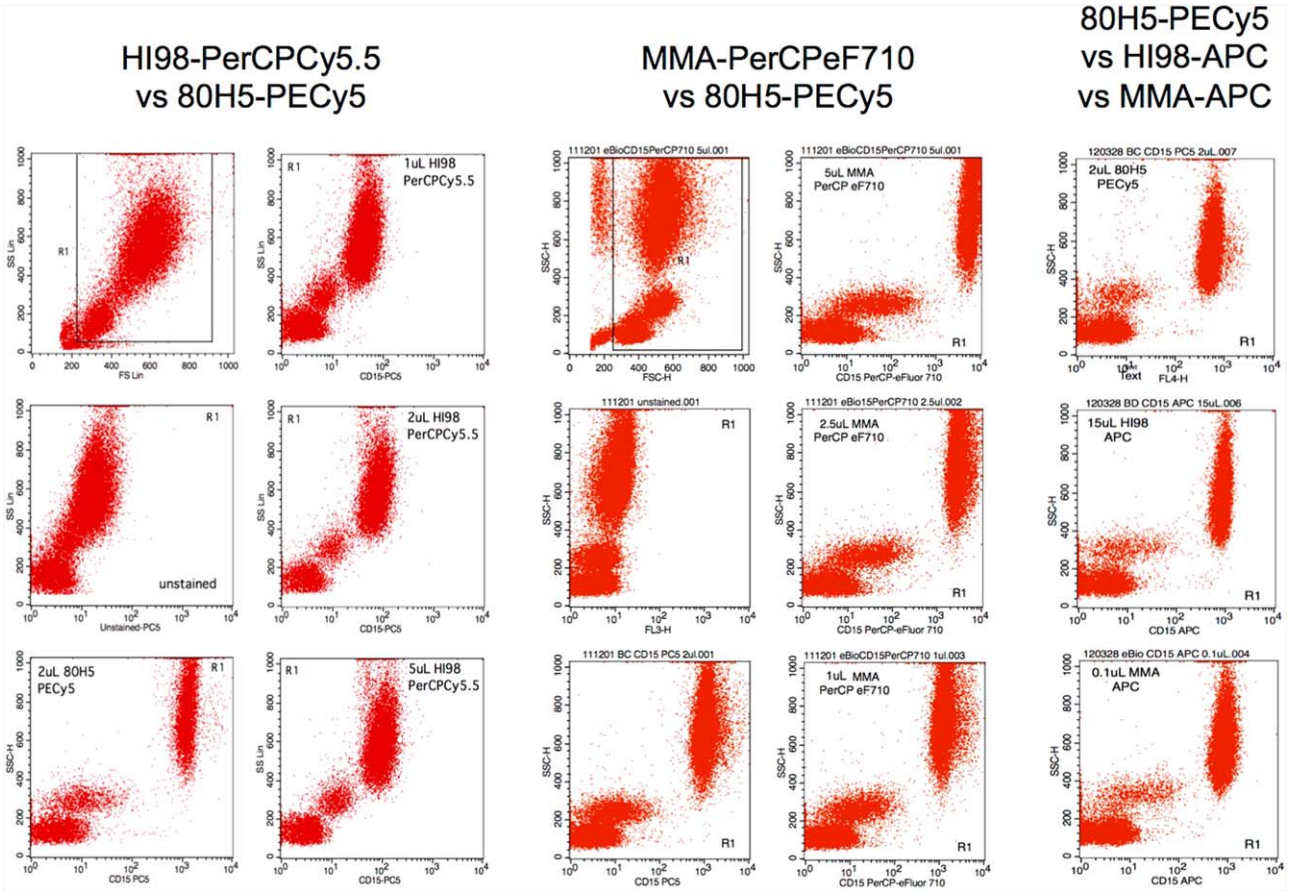

FIG. 8. Comparison of CD15 conjugates. Left panel: Unstained fresh normal PB sample post lysis and wash (top left). Debris excluded with region R1. R1 events displayed on SS versus unstained cells in PECy5 PMT (middle) and voltage adjusted to ensure lymphocytes are on scale. Bottom left plot, 2 μL CD15-PECy5 (clone 80H5, Beckman Coulter. Right panel top to bottom show titration of HI98-PerCPCy5.5 at 1 μL (top), 2 μL (middle) and 5 μL (bottom). Middle panel (right), top to bottom) shows a titration of CD15-PerCPeFluor710 (clone MMA, eBio/Thermo Fisher) at 5 μL (top), 2.5 μL (middle) and 1 μL (bottom). Middle panel bottom left shows 2 μL CD15-PECy5 (clone 80H5). Right panel shows preferred volumes of CD15 conjugates; top: 2 μL CD15- PECy5 (clone 80H5); Middle: 15 μL CD15-APC (clone HI98) and bottom: 0.1 μL CD15-APC (clone MMA, eBio/Thermo Fisher). [Color figure can be viewed at wileyonlinelibrary.com]

These assays and reagent sets were found to be highly reliable across FC500 and FACSCalibur instrument platforms in International studies performed under the auspices of the UK NEQAS organization using whole blood stabilized samples (30). Subsequently, 5-color single tube assays capable of simultaneous detection of both neutrophils and monocytes based on FLAER, CD157, CD15, CD64, and CD45 were developed for a variety of clinical cytometers with 5 or more PMTs (25–27), and again independently validated (31). Most recently, 6-color (26,34) assays have been developed for clinical instruments with 6 or more PMTs.

As indicated above, it is advisable to evaluate both neutrophils as well as monocytes as the latter may reflect the size of the PNH clone more accurately in some cases (see figure 12, Part 3, A. Illingworth et al. this issue). Lymphocytes that do not represent a suitable target cell-type for assessing the presence or extent of PNH disease because of their longevity; however they are extremely useful as "internal controls" to confirm optimal instrument setup/compensation as well as to monitor the performance of all antibody and FLAER conjugates used in the assay.

While PNH type II phenotypes can be detected in neutrophils and monocytes, the clinical significance if any, of such populations is unknown at this time. Even with carefully selected and validated reagent sets, it is clear for example, that CD157 generates different numbers of PNH type II neutrophils and PNH type II monocytes than could be delineated with either CD24 (for neutrophils) or CD14 (for monocytes).

Gating Antibodies for Neutrophils and Monocytes

CD45 for gating leukocytes

For reasons detailed above, CD45 is recommended as part of any gating strategy to identify specific subsets of leukocytes. The pan–CD45 conjugate to be used needs to be titrated as using saturating amounts of this reagent will push many leukocytes off-scale if the PMTv are set with unstained lymphocytes. As with CD235a and CD59 in normal samples, there are no internal negative

populations that can be used to perform S:N and SI calculations. Setting the PMTv with unstained cells in which the CD45 conjugate will be detected will generally lead to the stained sample requiring very little antibody before the cells are too bright to visualize on scale. This can lead to the use of insufficient CD45 conjugate that can become problematic if subsequently, a highly cellular sample is encountered. While it could be advisable in this setting to add some unlabeled CD45 to maintain "close to saturation" staining levels without pushing the stained cells off-scale, an easier way to deal with this for CD45 at least is to adjust the CD45 PMTv down a little so that lymphocytes in the stained sample are visualized at around the third decade of fluorescence when sufficient CD45 conjugate has been used.

CD15 gating for neutrophils

As indicated above, CD15 is recommended for gating neutrophils (11,24–27,29–32). After testing a large number of CD15 clones in a variety of conjugated forms, clones 80H5 (Beckman Coulter) and MMA (obtained from eBioscience) and MEM-158 (EXBIO) have been found to be reliable reagents to delineate neutrophils from monocytes and lymphocytes. As most CD15 clones are of the IgM subclass, neutrophil aggregation can be a serious issue at anything approaching antibody saturation and extensive titration may be needed to optimize the volume to use. In general terms, once optimally titrated, a "good" CD15 conjugate should separate neutrophils from monocytes by at least 1 log of fluorescence and in this setting, the separation between CD15-stained neutrophils versus unstained lymphocytes would be in excess of 1 log difference. As shown in Figure 8, left panel, compared to the 80H5–PC5 conjugate at 2 μL, the HI98-PerCP-Cy5.5 reagent (BD Biosciences) is incapable of effectively delineating neutrophils from monocytes regardless of reagent volume used, potentially leading to the generation of misleading results if reagents such as CD24 are used to detect GPI-deficient neutrophils. In Figure 8, middle panel, the MMA–PerCP–eFluor710 conjugate is as effective at 1 μL as 2 μL of the predicate 80H5–PC5 conjugate. In antibody validation studies performed prior to the UK NEQAS study (30), we found that 15 μL of the APC conjugate of HI98 was required to generate a >1 log difference between stained neutrophils and monocytes and generated similar results to the predicate 2 μL of 80H5-PC5 (Fig. 8, right panel). However, extensive titration of the MMA–APC conjugate was required to get the stained neutrophils on scale. While 0.1 μL was sufficient for this conjugate to reliably separate neutrophils from monocytes, at this dilution, the antibody is considerably under saturating, and in this setting, it would be advisable to add unlabeled MMA to the conjugate to maintain close-to-saturation staining levels. Extensive titration was also needed to optimize the staining of MEM-158 in PerCPCy5.5 conjugated form, to obviate the generation of neutrophil aggregates at staining volumes of 0.25 μL or higher (34).

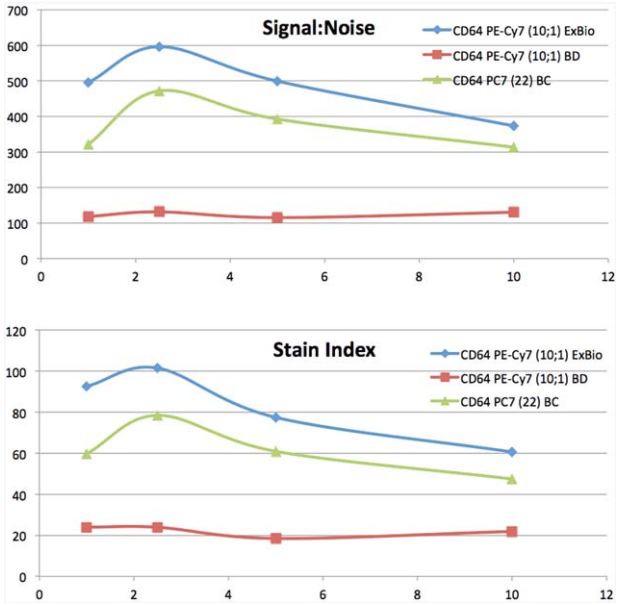

FIG. 9. Comparison of 3 CD64–PECy7 conjugates. Graphs show signal-to-noise ratio (SN) and staining index (SI) calculations from titrations of the EXBIO-sourced 10.1 conjugate (top), BD Biosciences-sourced 10.1 conjugate (bottom), and Beckman Coulter-sourced clone 22 (middle). [Color figure can be viewed at wileyonlinelibrary.com]

CD64 gating for monocytes

As indicated above, CD64 is recommended for gating monocytes (11,24–30). After testing a number of CD64 clones in a variety of conjugated forms, clones 22 (Beckman Coulter) and 10.1 (EXBIO) have been found to be reliable reagents to delineate monocytes from neutrophils and lymphocytes. As shown in Figure 9, in which we compared the S:N and SI of 3 commercial variants of PE–Cy7 conjugates of CD64 clones, the EXBIO 10.1 (blue graph) appeared to be marginally superior to the clone 22 conjugate (Beckman Coulter, green graph). Both clone 22 and the EXBIO 10.1 conjugate also exhibited very good staining characteristics offering greater than a 1.5-log separation between stained monocytes in the internal control lymphocyte population at all points in the titration (Fig. 10, top two rows). In contrast, the 10.1 conjugate from BD (Fig. 9, red graph) exhibited significant background staining of lymphocytes with increasing volumes of reagent (Fig. 10, bottom row) and the specific staining of monocytes fell below the minimum 1-log separation from the internal lymphocyte control population. The dot plots from all 12 data files shown on Figure 10 confirm that 2.5 μL of either 10.1 (EXBIO) or clone 22 (Beckman Coulter) would be close to the optimal titration point for gating monocytes for subsequent detection of PNH monocytes. In previously published studies, we have found clone 22 reagent to be very reliable in a variety of other conjugated forms including PE–Cy5 and ECD on Beckman FC500 and

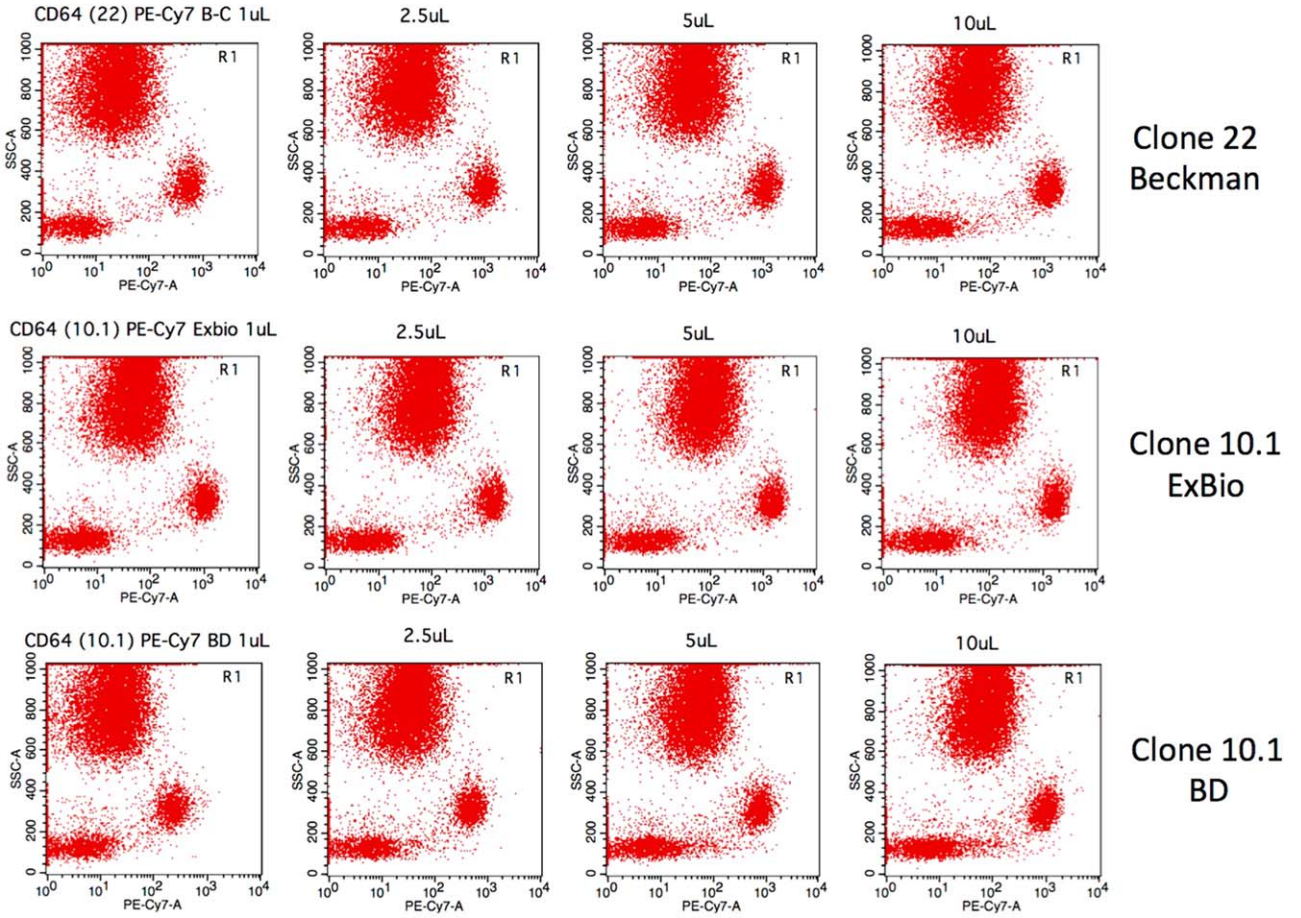

FIG. 10. Comparison of CD64–PECy7 conjugates. Dot-plot representation of CD64–PECy7 titrations shown in Figure 9. Data confirm the superiority of the EXBIO 10.1 and Beckman Clone 22 conjugates and also confirms optimal volume of each reagent at about 2.5 μL. [Color figure can be viewed at wileyonlinelibrary.com]

Navios instruments, while the APC conjugate of 10.1 (BD) was also suitable in a 4-color FLAER/CD24 assay for the FACSCalibur, and for 5-color FLAER/CD157-based assays on Canto II cytometers (11,24–26,30).

GPI-Linked Reagents for Neutrophils and Monocytes

FLAER for neutrophils and monocytes

As described in the original ICCS Guidelines (19) and emphasized in several subsequent publications (11,24–32) at least two GPI-linked structures per WBC lineage must be assessed and whenever possible, one should be FLAER, due to the latter's ability to detect a wide range of GPI-linked structures on a wide range of white blood cell types. This use of 2 GPI-linked structures per lineage approach gives increased robustness to the assay and significantly increases accuracy and sensitivity. For neutrophils, combinations of FLAER-Alexa488 with CD24-PE or FLAER-Alexa488 with CD157-PE represent the most validated and most widely deployed combinations. Similarly, FLAER can be used in combination with CD14-PE or with CD157-PE to detect GPI-deficient monocytes. Note that other than FLAER, that is only available as an Alexa488 conjugate, good assay design suggests that the other target structures in the assay, namely, the GPI-linked structures, be detected by antibodies conjugated to the brightest nontandem fluorochromes such as phycoerythrin (PE) and/or allophycocyanin (APC).

FLAER is nowadays marketed as a liquid preparation (Cedarlane) and so can be treated essentially the same way as any other antibody conjugate. Just like the optimal means to assess CD59 conjugates requires the availability of a PNH sample with a well-defined PNH type III population (see above), to perform a proper titration of FLAER for analysis by S:N and SI assessment requires the availability of a PNH sample that ideally contains a readily detectable population of PNH type III neutrophils as well as a readily detectable normal neutrophil population. The use of a counterstain such as CD45 helps to delineate neutrophils from other events to ensure clean data can be generated from the titration of the FLAER reagent. This is the approach taken in Figure 11 in which a PNH sample with a well-defined PNH type III neutrophil population (~40%) was stained with CD45–405 M (clone YAML 501.4, Cedarlane) that excites off

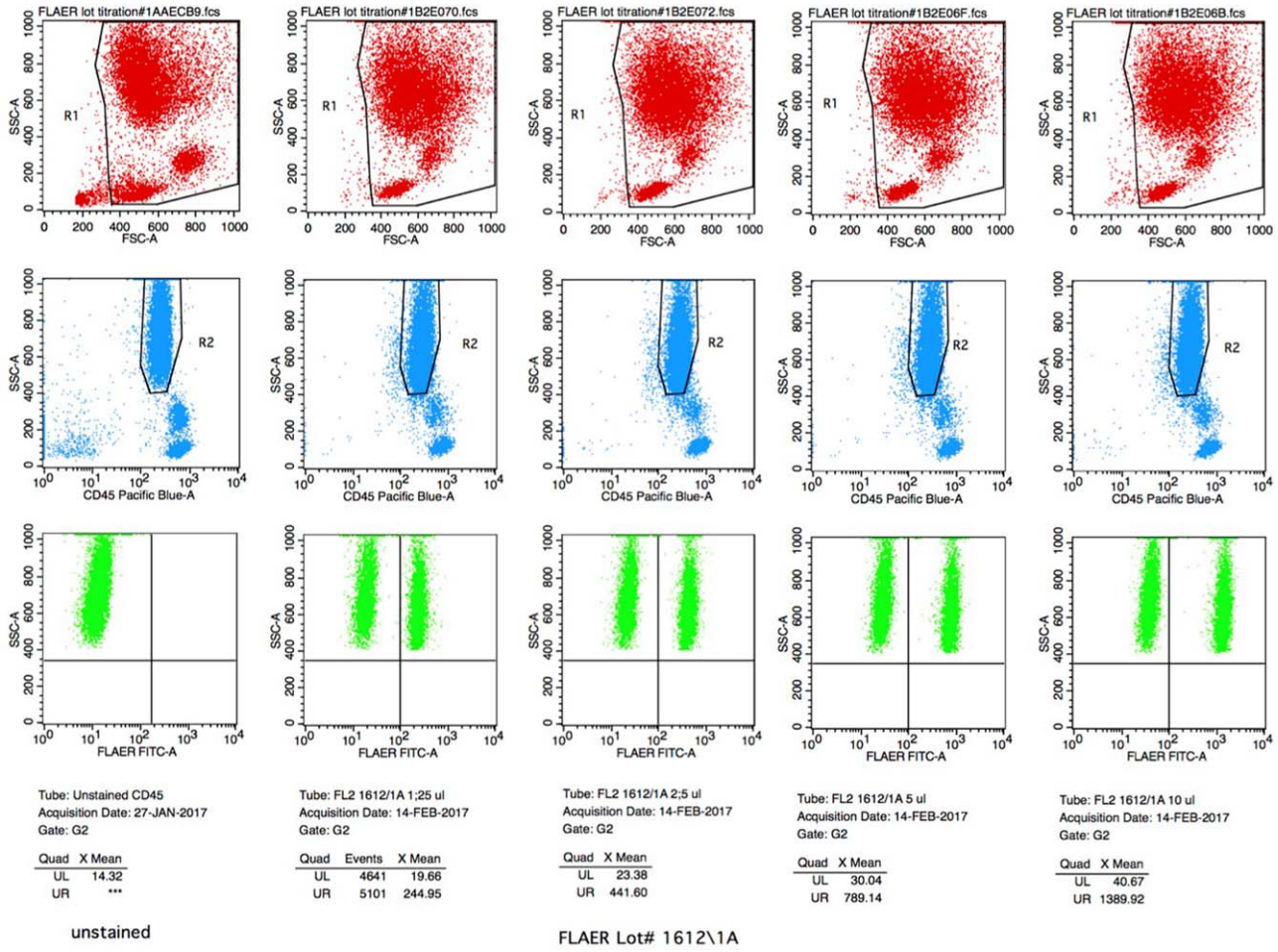

FIG. 11. Titration of FLAER-Alexa488. Fresh PNH sample with 45% PNH neutrophil clone stained with a combination of 2.5 μL CD45–405 M (clone YAML 501.4, Cedarlane) and various volumes of FLAER-Alexa488 (Cedarlane). Analysis of dot plots establish 3–5 μL as the optimal volume to use per 100 μL of blood sample, due to increasing background staining of PNH neutrophils beyond this concentration. [Color figure can be viewed at wileyonlinelibrary.com]

the 405 nm laser into the 450/40 nm (Pacific Blue) PMT. The titration of FLAER (bottom row) shows the optimal volume of FLAER/100 μL of blood sample to be in the 3–5 μL per test range. At this volume, the separation of normal from PNH neutrophils comfortably exceeds 1 log. Although the S:N and SI calculations (not shown) suggest using a larger volume of FLAER would be optimal, the increased non-specific staining of the PNH neutrophils at 10 μL/test shown in Figure 11 suggests using 2.5–5 μL of this specific lot.

CD24 for neutrophils

For CD24, we have tested a number of clones/conjugates and clones ALB9 (Beckman Coulter), SN3 (obtained from eBioscience) and ML5 (BD) perform adequately with all conjugates showing a >1-log difference in fluorescence between stained neutrophils and unstained internal control T lymphocytes across the titration curve (Fig. 12, left and middle panels respectively). While these reagents have been used in earlier studies in 4-color (neutrophils only) and 6-color (single tube neutrophils/monocyte) combinations, the SN3 conjugate (Fig. 12, right panel) is clearly superior requiring only around 2 μL to effect excellent separation of neutrophils from monocytes and unstained T-lymphocytes. Interestingly, the ability of the SN3 conjugate to delineate stained B-lymphocytes from unstained T-lymphocytes was also better than for the other two clones tested. Assessment of the SN3 CD24–APC conjugate (used in some other combined neutrophil/monocyte reagent sets) has confirmed the SN3–APC conjugates (eBioscience, EXBIO) to also be superior to other APC-conjugated clones that we have tested (data not shown). Note: SN3-PE can also be obtained from Thermo Fisher in ASR (analyte specific reagent) format but performs less well compared with the ASR and RUO (research use only) formats originally available from eBioscience (Illingworth A., unpublished observations). The latter underscore the critical need to test and validate all reagents prior to deployment in clinical laboratory.

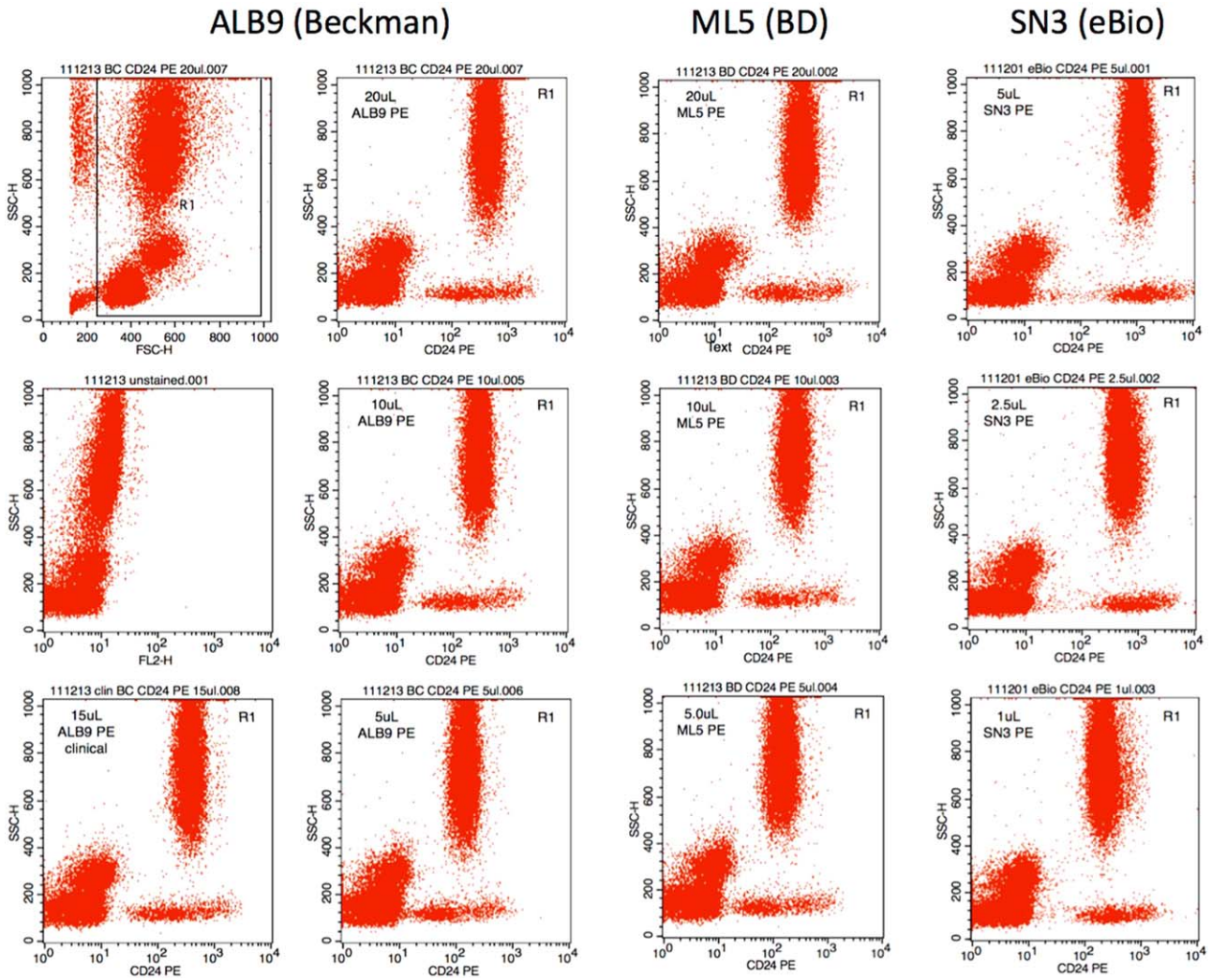

FIG. 12. Titration of 3 CD24–PE conjugates. Left panel, left column shows light scatter (top) and PMT setting (using unstained cells), and in-house ALB9 conjugate (Beckman Coulter) at 15 μL per test. Left panel right column shows the titration of ALB9-PE. The middle panel shows the titration of ML5-PE clone (BD Biosciences) and the right panel shows the titration of the SN3–PE conjugate (eBio/Thermo-Fisher). [Color figure can be viewed at wileyonlinelibrary.com]

CD14 for monocytes

For CD14, we have tested PE conjugates of a number of clones and while RMO2-PE (Beckman) performs adequately at 5 μL per test as described previously (11,24–26) (Fig. 13 middle panel), the MoP9-PE (BD Biosciences) at 5 μL the target monocyte population even better while the internal control lymphocyte population remained unstained at that volume (left panel). Both PE conjugates showed an almost 2-log separation between stained monocytes and unstained internal control lymphocyte populations across the titration. Unstained cells are shown in the far left panel to show the gating strategy employed. Unstained cells (top left) were displayed to gate and exclude debris (R1). Cells from R1 were displayed to optimize PMT setting to get lymphocytes on scale (middle plot). Bottom left plot shows staining of predicate (Beckam Coulter) RMO2–PE conjugate for comparison. However the best CD14–PE conjugate of those tested was the 61D3 clone (eBioscience) (right panel). Although the internal control lymphocyte population remained unstained at 5 μL, and S:N and SI calculations (not shown) suggested to use up to 5 μL of this reagent, the intensity of the stained monocytes was beyond optimal, almost going off-scale at this volume of reagent. Simple observation of the dot plots suggested 1 μL of 61D3 to be optimal for 100 μL of test (normal blood) sample. Beyond what is shown in Figure 13, the Tuk4–PE conjugate (Invitrogen/Thermo Fisher, A Illingworth unpublished data) and the RMO2 clone in APCA700-conjugated form (Fig. 15) have been shown to perform very well, as has the APCeFluor780 conjugate of 61D3 (eBioscience, see Case 14 of Part 3, Illingworth A et al. this issue).

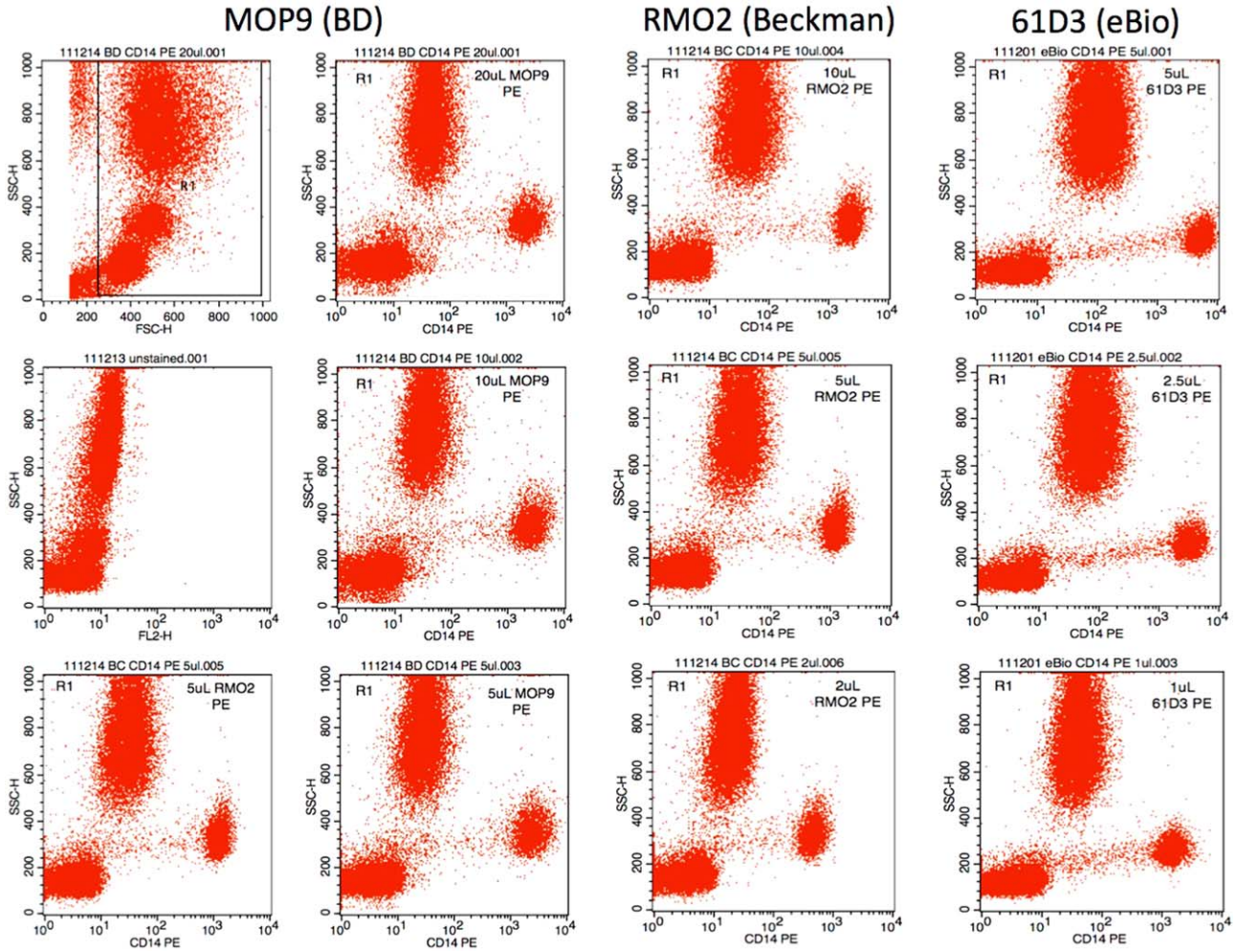

FIG. 13. Comparison of CD14–PE conjugates. Light scatter and PMT settings were optimized as described in legends to Figures 8 and 12 for the PE detector. See text for details. [Color figure can be viewed at wileyonlinelibrary.com]

CD157 for neutrophils and monocytes

CD157 (33) is a GPI-linked structure brightly expressed on both neutrophils and monocytes (12,13,25–27) and for cytometers with 5 or more PMTs can be used in 5-color combination with FLAER instead of CD24 and CD14 for single tube high-sensitivity assessment of GPI-deficient neutrophils and monocytes (25–27,31,32). Only two CD157 clones, SY11B5 (eBioscience) and RF3 (Beckman Coulter) have been available to date and in parts of the world, only the SY11B5 was initially available in PE-conjugated ASR format. The RF3–FITC conjugate was significantly less effective than the PE-conjugated SY11B5 clone (DRS unpublished). Where the PE version of RF3 is available, comparative studies with the SY11B5 clone have shown the latter to significantly outperform the RF3 clone (D. Payne et al. unpublished). Very recently, SY11B5-PE has become available from a variety of other vendors in either RUO format (BD Biosciences, Sysmex) or ASR format (EXBIO, Beckman Coulter).

Sample staining and data acquisition

Anticoagulated whole peripheral blood is used for the WBC assays. Bone marrow, although it can be analyzed using modern WBC assays is not recommended due to the presence of immature mono-myeloid cells that do express some GPI-linked antigens including CD14 at very low levels. Blood should be fresh, although if stored after draw at 4°C, has been validated to be stable beyond 48 h for WBC and RBC analysis. Reverse-pipetting is used to dispense 100 μL of sample into the staining tube, taking all the precautions noted above for the RBC assay to avoid aerosols and ensure that sample is not left on the inside wall of the tube. The reagent set in use should be cocktailed, once optimal volumes of each reagent have been determined by titrations. The appropriate volume of cocktail is added directly into the blood sample at the bottom of the tube and gently admixed by up-and-down pipetting. After gentle swirling to avoid aerosols, the sample is incubated in the dark for 20–30 min at room temperature before RBC lysis.

Table 2
Recommended Clones/Conjugates and Reagent Cocktails to Determine High-Sensitivity Detection of PNH WBC on Beckman Coulter Cytometers

4-C Neutrophils[A]	FLAER CD24-PE	CD15-PC5	CD45-PC7
4-C Monocytes[A]	FLAER CD14-PE	CD64-PC5	CD45-PC7
5-C Neuts+Monos[AB]	FLAER CD157-PE CD64-ECD	CD15-PC5	CD45-PC7
6-C Neuts+Monos[B]	FLAER CD24-PE	CD15-PC5	CD64-PC7 CD14-APC700 CD45-KO

Target	Antibody conjugates	Purpose	Clone (Vendor)
WBC	FLAER-Alexa488	GPI-linked (Neuts and Monos)	NA (Cedarlane)
	CD24-PE (4C 6C)	GPI-linked (Neuts)	SN3 (eBio), ALB9 (BC)
	CD14-PE (4C)	GPI-linked (Monos)	61D3 (eBio), RMO52 (BC), Tuk4 (Invitrogen)
	CD14-APC700 (6C)		RMO52 (BC)
	CD157-PE (5C)	GPI-linked (Neuts + Monos)	SY11B5 (eBio, BC, EXBIO, BD, Sysmex)
	CD64-PC5 (4C)	Gating on Monocytes	Clone 22 (BC)
	CD64-ECD (5C)		Clone 22 (BC)
	CD64-PC7 (6C)		Clone 22 (BC), 10.1 (EXBIO)
	CD15-PC5 (4C 5C)	Gating on Neutrophils	80H5 (BC)
	CD45-PC7 (4C 5C)	Debris and unlysed RBC	J33 (BC)
	CD45-KO (6C)	exclusion, pattern recognition	J33 (BC)

A: For 1-laser FC500; B: For 3-laser Navios.

There are a variety of commercial lysing/fixing agents available such as VersaLyse, FACSLyse and ImmunoPrep and most perform adequately by showing good separation between the WBC populations. After lysis following manufacturers' recommendations, the sample is centrifuged, and washed with phosphate buffered saline supplemented with 1% bovine serum albumin (BSA). The sample is resuspended in 0.5–1 mL of PBS and acquired. In general, 50,000 CD15-gated neutrophils should be collected, but if small numbers of PNH phenotypes are detected, additional data will need to be collected from essentially the entire sample (discussed in greater detail in Part 3, Illingworth A. et al., this issue). In neutropenic samples, it may be necessary to stain a larger volume of sample, using an increased volume of reagent cocktail. As described elsewhere (11,24–27), this high sensitivity neutrophil assay has been shown to be sensitive to the 0.02%–0.05% level in general use and in specific studies, can detect as few as 10 clustered PNH phenotypes in 500,000 events, with a background rate on normal samples of between 4 and 10 per million.

Reagent cocktails for 4-, 5-, and 6-color WBC assays

4-color assays. Before addressing specific reagent cocktails, it should be noted that cocktailing of reagents is very important for WBC assays, just as it is for the RBC assay. If reagents are added one at a time, there is increased risk of failing to add individual reagents, or adding too much or too little, especially where very small volumes of some individual reagents need to be accurately pipetted. Such errors run the risk of generating 'laboratory-induced PNH' if the GPI-specific reagents are inadvertently omitted. For laboratories that run the assay infrequently, there is an increased requirement to ensure that the reagents used in the assay are working as specified. In this setting, it is recommended that the laboratory stain a normal sample at least once per month to validate the performance of all reagents used in the assay (11,24–26).

The Practical Guidelines (11) defined two 4-color reagent cocktails for the high sensitivity detection of PNH neutrophils and PNH monocytes. The neutrophil assay was based upon a cocktail of FLAER, CD24-PE, CD15-PC5, and CD45-PC7 (Beckman FC500) or FLAER, CD24-PE, CD45-PerCP, and CD15-APC (BD Biosciences FACSCalibur).

The monocyte tube that was set up only when the RBC and neutrophil assays indicated the presence of PNH phenotypes above background rates used a cocktail of FLAER, CD14-PE, CD64-PC5, and CD45-PC7 (FC500/Navios), or FLAER, CD14-PE, CD45-PerCP, and CD64-APC (FACSCalibur/Canto). Examples of the Boolean gating strategies used in each assay on FC500 platform are shown in figures 2 and 3 of ref. 11. In brief, a light scatter plot and a CD45 versus SSC plot are used to remove debris and any unlysed RBCs, respectively. CD45+ events are displayed on a CD15 versus SSC plot and neutrophils are gated. Gated neutrophils are then displayed on an FLAER versus CD24-PE plot and GPI-deficient neutrophils can be gated and quantified as a percentage of the CD15-gated neutrophils. The strategy for identifying PNH monocytes follows a similar analytic pattern, except that CD64 is used (in place of CD15) to gate monocytes and PNH monocytes are quantified by the lack of FLAER and CD14-PE binding. In both assays, the lymphocytes serve as internal controls to demonstrate appropriate staining patterns for the reagents in use.

Subsequent studies using these assays/reagent sets have shown them to be reproducible and robust using fresh samples across multiple laboratories (29) and in International studies performed with stabilized whole blood samples under the auspices of the UK NEQAS organization (30). The specific reagent cocktails, recommended clones and recommended conjugated forms used in these studies

Table 3
Recommended Clones/Conjugates and Reagent Cocktails to Determine High-Sensitivity Detection of PNH WBC on Becton Dickinson Biosciences Cytometers

4-C Neutrophils[A]	FLAER	CD24-PE	CD45-PCP	CD15-APC	
4-C Monocytes[A]	FLAER	CD14-PE	CD45-PCP	CD64-APC	
5-C Neuts+Monos[B]	FLAER	CD157-PE	CD15-PCPeF710 (or PCPCy5.5)	CD64-APC	CD45-eF450
5-C Neuts+Monos[C]	FLAER	CD157-PE	CD15-PCPeF710	CD64-APC	CD45-APCH7
6-C Neuts+Monos[B]	FLAER	CD24-PE	CD15-PCPeF710 CD64-PECy7	CD14-APC	CD45-eF450

Target	Antibody conjugates	Purpose	Clone (vendor)
WBC	FLAER-Alexa488	GPI-linked	NA (Cedarlane)
	CD24-PE (4C 6C)	GPI-linked (Neuts)	SN3 (eBio), ML5 (BD)
	CD14-PE (4C)	GPI-linked (Monos)	61D3 (eBio), MoP9 (BD) TUK4 (Invitrogen)
	CD14-APC (6C)		
	CD157-PE (5C)	GPI-linked (Neuts + Monos)	SY11B5 (eBio, EXBIO, BD, Sysmex, BC)
	CD64-APC (4C 5C)	Gating on Monocytes	10.1 (BD, eBio)
	CD64-PECy7 (6C)		10.1 (EXBIO), 22 (BC)
	CD15-APC (4C)	Gating on Neutrophils	HI98 (BD)
	CD15-PerCPCy5.5 (5C 6C)		MEM-158 (EXBIO)
	CD15-PerCPeFluor710[BC]		MMA (eBio)
	CD45-PerCP (4C)	Debris, unlysed RBC exclusion,	2D1 (eBio)
	CD45-eF450 (5C 6C)[B]	pattern recognition	2D1 (eBio)
	CD45-APCH7 (5C)[C]		2D1 (BD)

A: For 2-laser FACSCalibur; B: For 3-laser FACSCanto; C: For 2-laser FACSCanto.

are shown in Tables 2 (for Beckman cytometers) and Table 3 (for BD Biosciences instruments).

5-color assays. The availability of a bright PE conjugate of the SY11B5 CD157 clone allowed the comparison of CD157-based 4-color neutrophil and monocyte assays with their CD24- and CD14-based predicates and data generated showed a very high correlation ($r^2 = 0.999$) across both assays. By replacing CD24-PE and CD14-PE with a single CD157-PE conjugate, we were able to develop a 5-color, single tube, high-sensitivity assay for both neutrophils and monocytes (25–27) that could be performed on the FC500, Navios, and Canto II instruments. This assay comprised of FLAER, CD157-PE, CD64-ECD, CD15-PC5, and CD45-PC7 (FC500 and Navios) or FLAER, CD157-PE, CD15-PerCPeFluor710, CD64-APC, and CD45-eFluor450 (Canto II). The latter can also be used on a Navios cytometer as it employs reagents that can be used across instrument platforms without modification (34). The 5-C assays had closely similar sensitivities to the original 4-color assays with similar very low background rates on 10 normal samples (27).

The gating strategy used for the 5-color FC500/Navios assays is shown in figure 5 of ref. 27, while a 5-C assay (FLAER, CD157-PE, CD15-PerCPeFluor710, CD64-APC, and CD45-eFluor450) capable of deployment on either Canto II or Navios instruments is shown in Figure 14. For laboratories equipped with both platforms, this allows a sample to be stained once and analyzed on either Navios or Canto instruments.

The 5-color assays are also robust and reproducible as evidenced by other studies comparing a 6-color (FLAER, CD24, CD14, CD15, CD64, and CD45) cocktail with the CD157-based 5-color assay (31).

Specific reagent cocktails, recommended clones and recommended conjugated forms used in the 5-color studies are shown in Table 2 (for Beckman cytometers), Table 3 (for BD Biosciences instruments), and Table 4 for cross-platform assays.

It should be noted that the neutrophils and monocytes of occasional non-PNH patient samples analyzed with CD157/FLAER based assays fail to express CD157. In some samples, CD157 is lost from the entire neutrophil and monocyte populations while in others, CD157+ and CD157− subsets of both lineages are observed (DRS unpublished observations). While the biological cause of this remains under study, the mandatory inclusion of the second GPI reagent (FLAER) is part of the built-in robustness of the assay, and prevents the misinterpretation of the data as a "PNH clone-containing sample"; the loss of two GPI-linked structures *per lineage* is required to identify *bona fide* PNH phenotypes. Furthermore, in keeping with current state-of-the-art guidelines (24–26), the RBC lineage was also analyzed and in every CD157- case there was no evidence of any PNH phenotypes confirming the finding as an isolated single (CD157) GPI deficiency. An example of a CD157-negative case (Case 14) using a 7-color combination (FLAER, CD157-PE, CD15-PerCPeFluor710, CD64-PECy7, CD24-APC, CD14-APCeFluor780, and CD45-eFluor450) is shown in Part 3 (A Illingworth et al. this issue). Such CD157-, non-PNH cases should simply be reported as 'No evidence of PNH in RBCs and WBCs'.

6-color assays. A number of 6-color, single tube, high-sensitivity assays have been developed whose gating strategies are also derived from the original 4- and 5-color assays.

In the example shown in Figure 15, a Navios-specific cocktail comprising FLAER, CD24-PE, CD15-PC5, CD64-PC7, CD14-APC700, and CD45-KO was used to stain a PNH sample. Neutrophils and

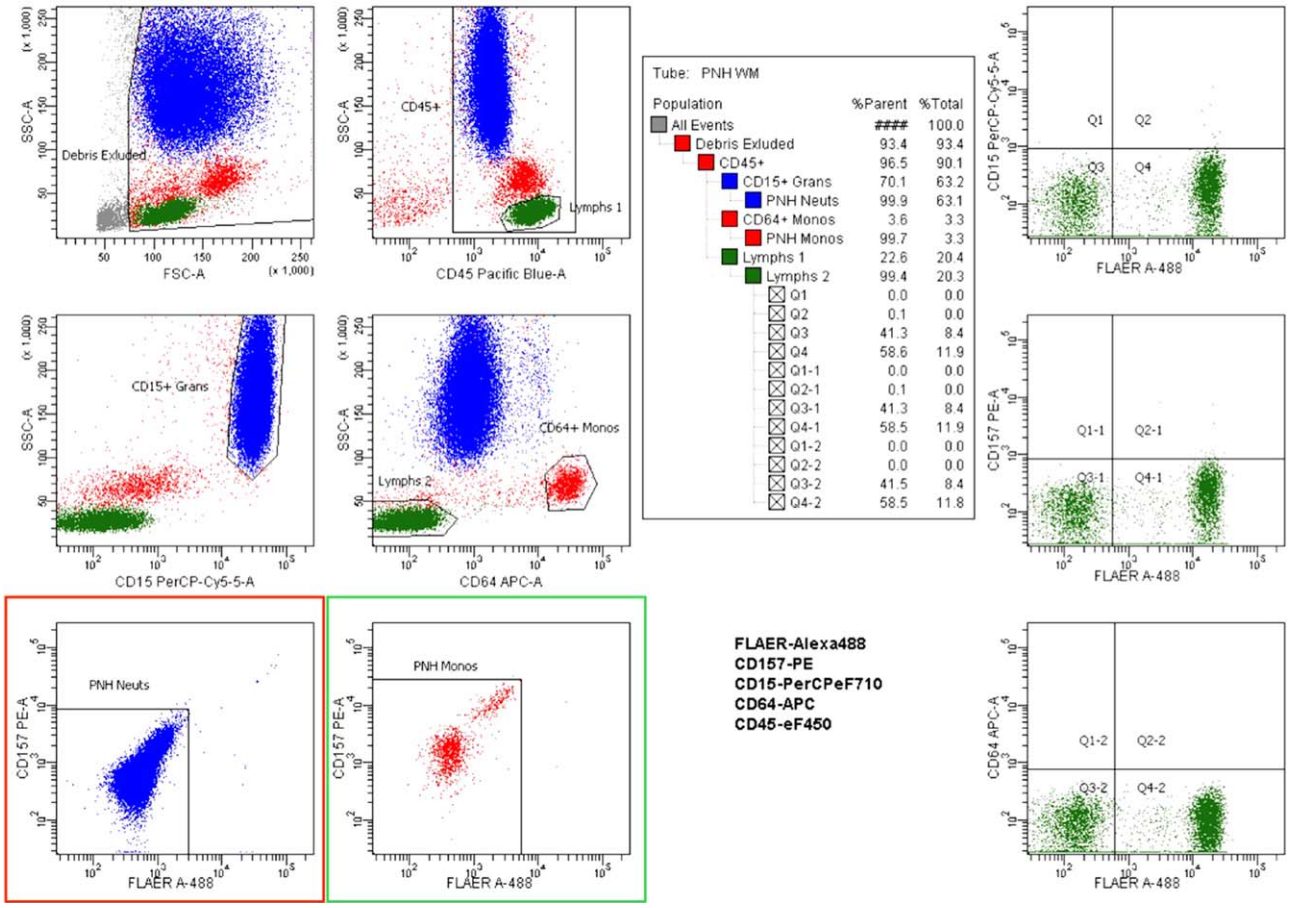

FIG. 14. Analysis of PNH sample with a 5-color combination of FLAER, CD157-PE, CD15-PerCPeFluor710, CD64-PECy7, and CD45-eFluor450 that can be used on 3-laser version of Canto and Navios platforms. Data shown are from a Canto II cytometer. Gating strategy performed as described in text. Data show essentially 100% PNH neutrophils (both Type III and Type II cells) and 100% PNH monocytes (both Type III and Type II cells). Sample also contains about 41.5% PNH lymphocytes. [Color figure can be viewed at wileyonlinelibrary.com]

monocytes are gated by a combination of light scatter, CD45 expression and either CD15 staining (neutrophils) or CD64 staining (monocytes). PNH phenotypes are detected and/or quantified based on their loss of FLAER and CD24 binding (neutrophils) or FLAER and CD14 binding (monocytes) (26).

Table 4
Recommended WBC Antibody Panels for Cross-Platform 3-Laser BC Navios/BD Canto: 5-, 6-, and 7-Color Assays

5-C Neuts+Monos	FLAER	CD157-PE	CD15-PCPT*	CD64-APC			CD45-eF450
6-C Neuts+Monos	FLAER	CD157-PE	CD15-PCPT*	CD64-PC7	CD24-APC		CD45-eF450
7-C Neuts+Monos	FLAER	CD157-PE	CD15-PCPT*	CD64-PC7	CD24-APC	CD14-APCeF780	CD45-eF450

Target	Antibody conjugates	Purpose	Clone (vendor)
WBC	FLAER-Alexa488 (all)	GPI-linked (Neuts + Monos)	NA (Cedarlane)
	CD157-PE (all)	GPI-linked (Neuts+ Monos)	SY11B5 (eBio, EXBIO, BD, BC)
	CD24-APC (6C 7C)	GPI-linked (Neuts)	SN3 (APC)
	CD14-APCeF780 (7C)	GPI-linked (Monos)	61D3 (eBio)
	CD64-APC (5C)	Gating on Monocytes	10.1 (BD)
	CD64-PC7 (6C 7C)	Gating on Monocytes	10.1 (EXBIO) or
		Gating on Monocytes	22 (BC)
	CD15-PerCPeF710 (all)	Gating on Neutrophils	MMA (eBio)
	CD15-PerCPCy5.5 (all)		MMA (EXBIO)
	CD15-PerCPCy5.5 (all)		MEM158 (EXBIO)
	CD45-eF450 (all)	Debris, unlysed RBC exclusion pattern recognition	2D1 (eBio)

T* = PerCP Tandems: PerCPeFluor710 or PerCPCy5.5.

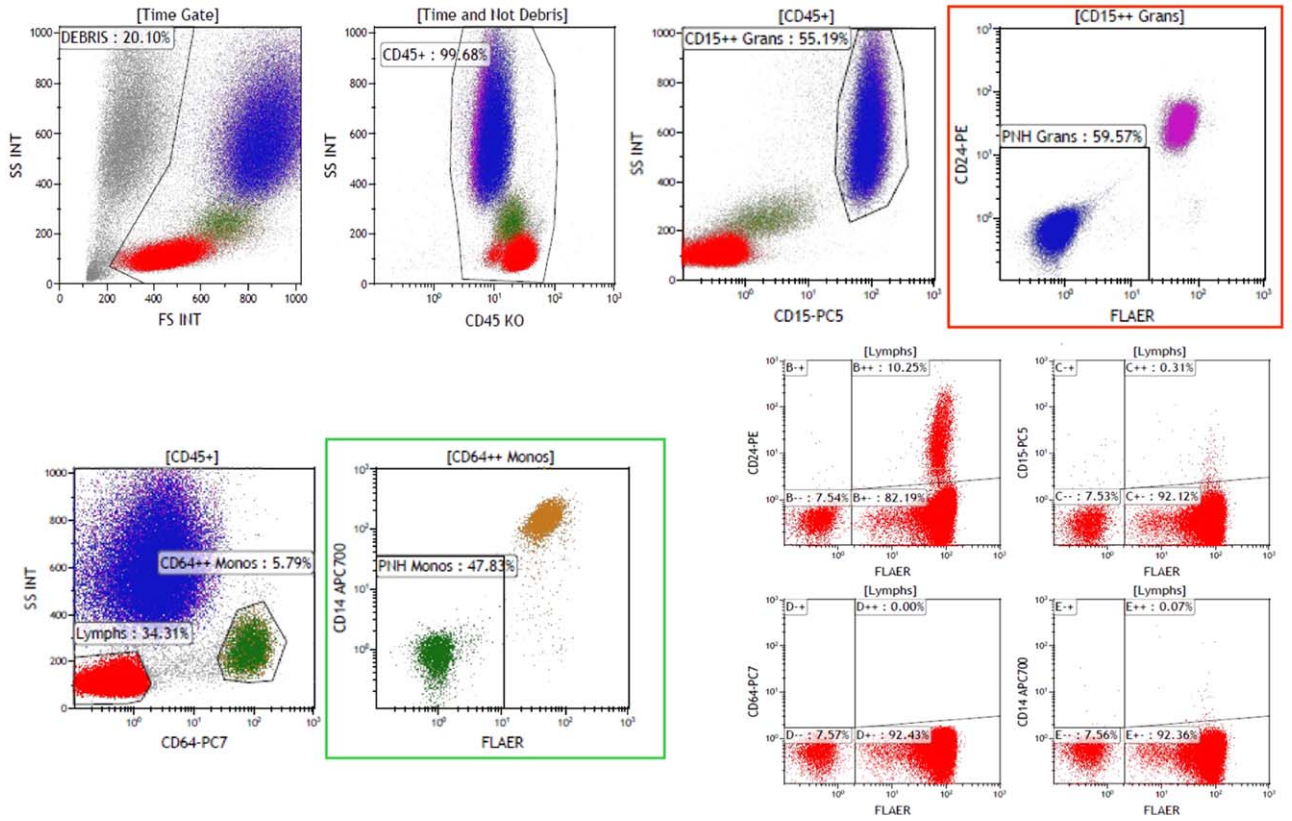

FIG. 15. Analysis of PNH sample with Navios-specific 6-color combination of FLAER, CD24-PE, CD15-PC5, CD64-PC7, CD14-APCA700, and CD45-KO. Gating strategy performed as described in text. Sample contains about 60% PNH neutrophils and about 48% PNH monocytes. Sample also contains about 7.5% PNH lymphocytes. [Color figure can be viewed at wileyonlinelibrary.com]

Other 6-color cocktails based on FLAER, CD24, and CD14 have been developed for the Canto II instruments, but depending on whether they are equipped with 2- or 3-lasers, the conjugated forms for the clones employed need to be chosen for each instrument (Table 3).

More recently, 6-color cocktails comprising FLAER, CD157-PE, CD15-PerCP-eFluor710, CD64-PECy7, CD24-APC, and CD45-eFluor450 have been developed that can be performed on both Navios and Canto II instruments (Fig. 16). Similar to the 5-C Navios/Canto II assay described above, samples can be stained with this 6-C reagent set and be analyzed on either/both Navios and 3-laser Canto variants.

Specific reagent cocktails, recommended clones and conjugated forms used in these 5C, 6C, and 7C cross-platform (3-laser Navios and Canto II instruments) assays are shown in Table 4. Detailed validation of WBC assays is described in Part 4 (Oldaker et al., this issue).

Alternative Approaches

Reticulocyte assays

In many cases, it is extremely difficult to delineate Type II RBCs from normal Type 1 cells even on two-dimensional plots. This problem can be significantly exacerbated when analyzing samples from recently transfused cases where transfused cells can express slightly lower levels of CD59 (SJ. Richards, personal communication). For these situations, the addition of CD71 can be used to identify and "gate" CD71-positive reticulocytes, thereby excluding RBCs (both transfused and recipient RBCs) and allowing an assessment of CD59 staining of the reticulocytes. A detailed protocol is provided elsewhere (25) and an illustration of the use of this assay in a transfused case can be found in the case study (16) in Part 3 (Illingworth A et al. this issue).

WBC assays

While we do not promote the use of non-FLAER-based approaches where FLAER is available and affordable, FLAER is not universally available and/or is too costly to obtain in some parts of the world. To address the feasibility of non-FLAER approaches, Marinov et al. performed a study to compare a predicate 5-color FLAER-CD157-based assay (31), with a 6-color non-FLAER assay containing CD15-FITC, CD157-PE, CD24-APC, CD14-APC-H7, CD64-PECy7, and CD45-PerCPCy7. The results confirmed very good agreement between the FLAER and

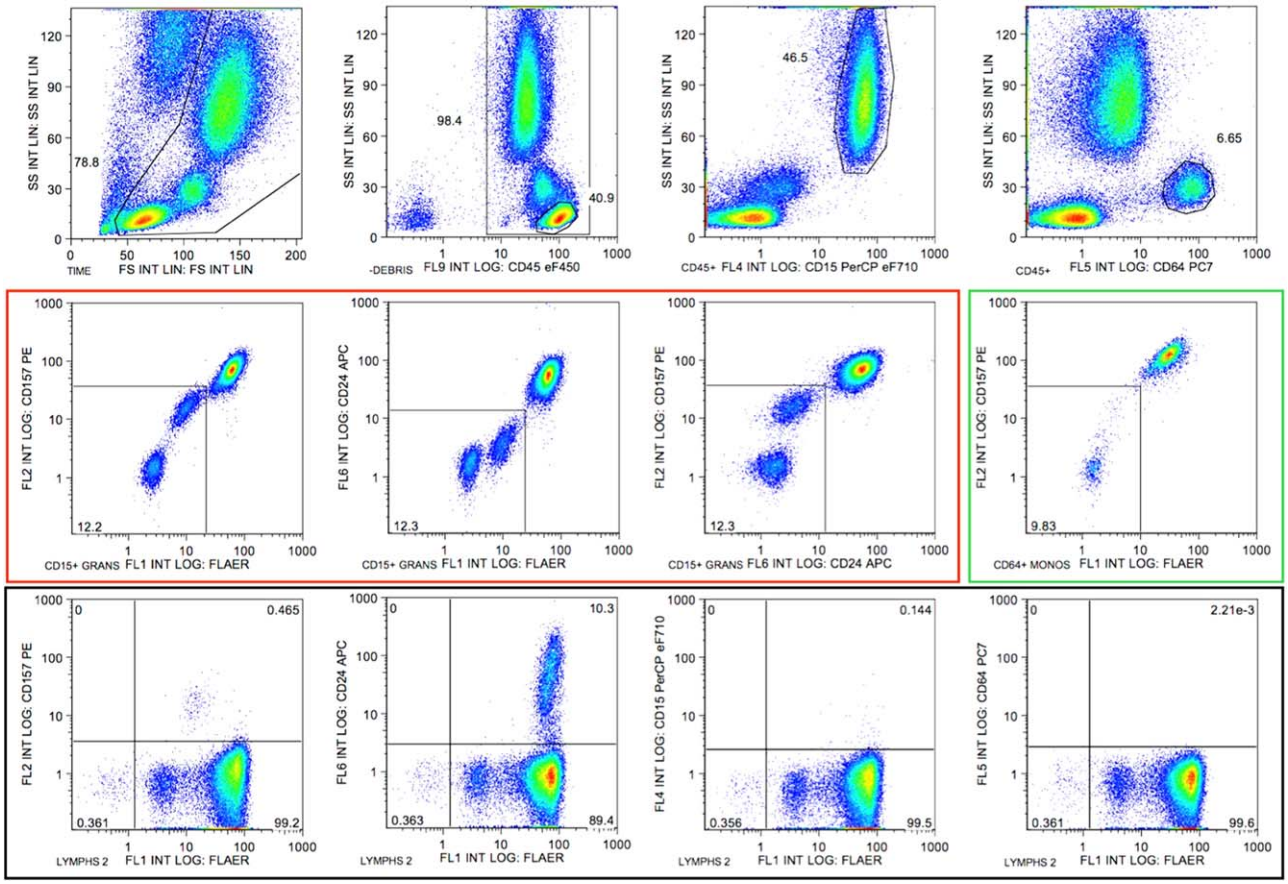

FIG. 16. Analysis of PNH sample with 6-color Navios/Canto II combination of FLAER, CD157-PE, CD15-PerCPeFluor710, CD64-PECy7, CD24-APC, and CD45- eFluor450. Gating strategy performed as described in text. Sample contains about 12.2% PNH neutrophils (both Type III and Type II cells) regardless of which pair (FLAER/CD157, FLAER/CD24, or CD157/CD24) of GPI-linked reagents is used to determine clone size (red box, middle row first three plots). Sample contains about 9.8% PNH monocytes (green box, middle row far right plot). Sample also contains about 0.36% PNH lymphocytes (black box, bottom row).

non-FLAER-based tubes, suggesting that a rapid and cost-effective simultaneous evaluation of PNH neutrophils and monocytes is possible without using FLAER (32).

While the Marinov approach (32) showed very good agreement between two separate methods (tubes), it is possible to do a more direct study using a 7-color assay comprising FLAER, CD157-PE, CD15-PerCP-eFluor710, CD64-PECy7, CD24-APC, CD14-APC-eFluor780, and CD45-eFluor450 in a single tube. As indicated above for certain 5-C and 6-C cocktails, it is possible to stain a sample once, but acquire/analyze on either Navios and/or Canto II instruments, with essentially identical data generated (34). With the 7-C approach, one can quantify the CD15-gated PNH neutrophil clone size using either FLAER and CD157, or FLAER and CD24, or CD157 versus CD24. In the same tube, one can quantify the CD64-gated PNH monocyte clone size using either FLAER and CD157, or FLAER and CD14, or CD157 and CD14. In this way, the FLAER-based analysis plots can be directly compared with the non-FLAER plots in each lineage within the same stained sample. Analysis of >40 PNH samples has shown that the FLAER-based plots derive virtually identical data to the non-FLAER plot for both neutrophil and monocyte lineages (34).

HIGH-SENSITIVITY DETECTION OF PNH PHENOTYPES BASED ON PUBLISHED GUIDELINES: SUMMARY

Red Blood Cells

Based on the extensive evaluation and validation of a large number of CD235a clones/conjugates and CD59 clones/conjugates, a simple and reliable highly sensitive 2-color flow assay was developed to detect PNH RBC phenotypes to a level of 0.002%. The assay could be performed on a variety of flow cytometric platforms regardless of manufacturer (Figs. 2 and 3). The background rate of PNH phenotypes in normal PB samples was in the 2–6 per million range (11,24–26). This assay is also very robust providing that labs deploying this assay adhere strictly to the use of the validated CD235a and CD59 conjugates shown in Table 1, and follow the simple staining and analytic procedures detailed elsewhere (11,24–26) and as outlined above. The assay has been validated by other groups (29,30).

White Blood Cells

Tables 2 and 3 show a list of recommended antibody conjugates and 4-, 5-, and 6-color combinations for Beckman Coulter cytometers (Table 2) and BD Biosciences cytometers (Table 3). Reagents presented in the tables underwent rigorous testing, on both normal and PNH samples before being admixed into cocktails and tested on a range of normal and PNH-containing samples. As detailed in Parts 3 and 4 (Illingworth A et al. and Oldaker T et al. this issue), the neutrophil assays described above have a lower limit of quantification (LLOQ) of about 0.02%–0.05% in day-to-day use, while the monocyte assays have a somewhat lower sensitivity based on the lower numbers of monocytes generally available for analysis. Background levels of PNH phenotypes in normal samples are also very low for both neutrophil and monocyte assays (11,24–27) and many of these WBC assays have also been validated in separate studies elsewhere (29–32).

ACKNOWLEDGMENTS

The authors thank Bruce Grieg, Ruud Hulspas, Michael Clemente, and Stephen Richards for reviewing the article. The authors thank Brian DuChateau (The Binding Site)/eBioscience), Miroslav Benko (EXBIO), Marsha Griffin (BD Biosciences), Eddie Johnson (Cedarlane Labs), and Ranbir Prah (Sysmex) for kindly donating some of the antibody conjugates used in this study. Some of the reagent testing and reagent cocktail validation studies outlined herein were supported by an Unrestricted Educational Grant from Alexion Canada.

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APPENDIX A

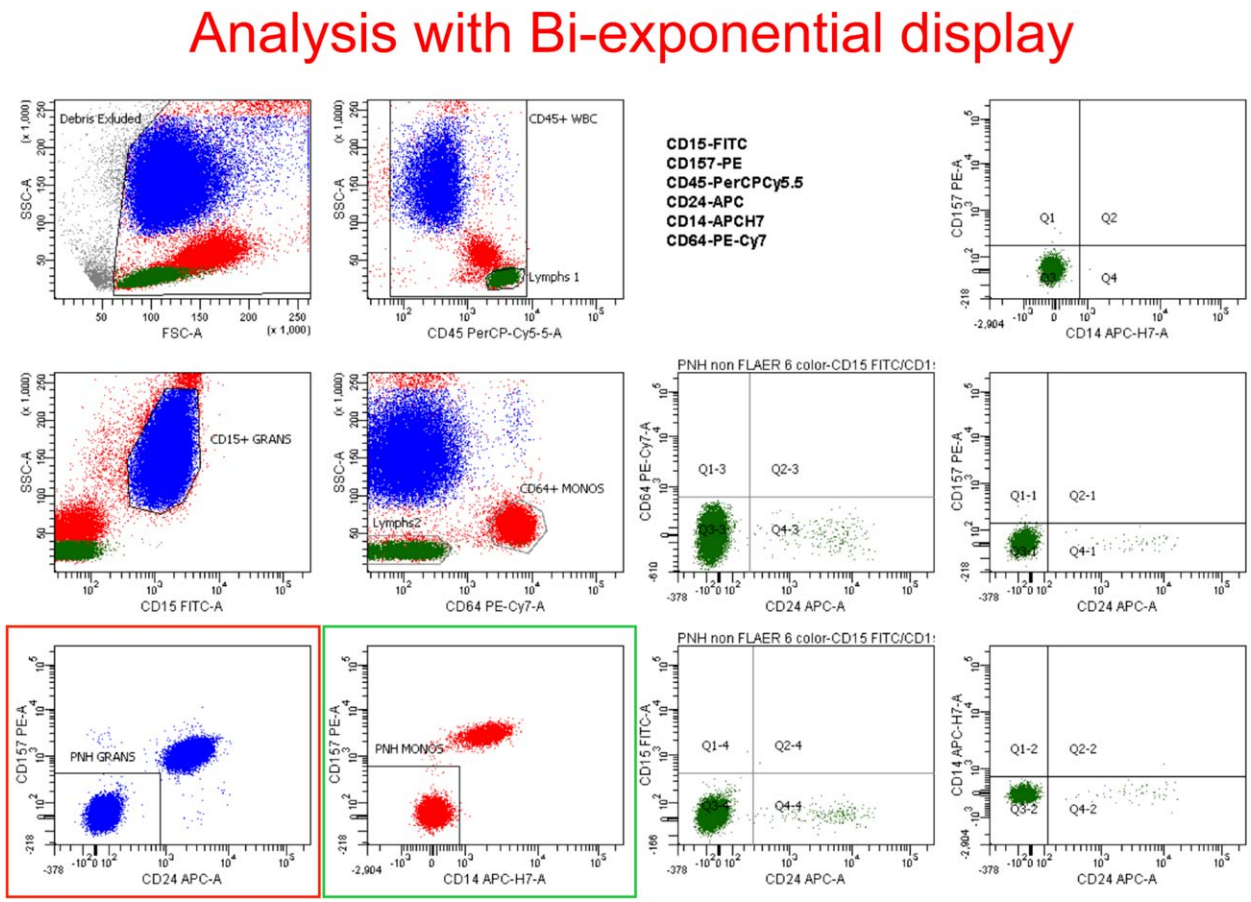

FIG. A1. Analysis of WBC assay with bi-exponential display. PB sample stained with CD15-FITC (clone MEM158), CD157-PE (clone SY11B5), CD45-PerCP-Cy5.5 (clone 2D1), CD64-PCy7 (clone 10.1), CD24-APC (clone SN3) and CD14-APC-H7 (clone MoP9). Data acquired on FACSCanto set up using manufacturer's generic setup/compensation strategies. It is possible to see all leukocyte subsets of interest only if the bi-exponential display is employed, showing that many of the "negative" events of interest, i.e., PNH phenotypes, are below zero. [Color figure can be viewed at wileyonlinelibrary.com]

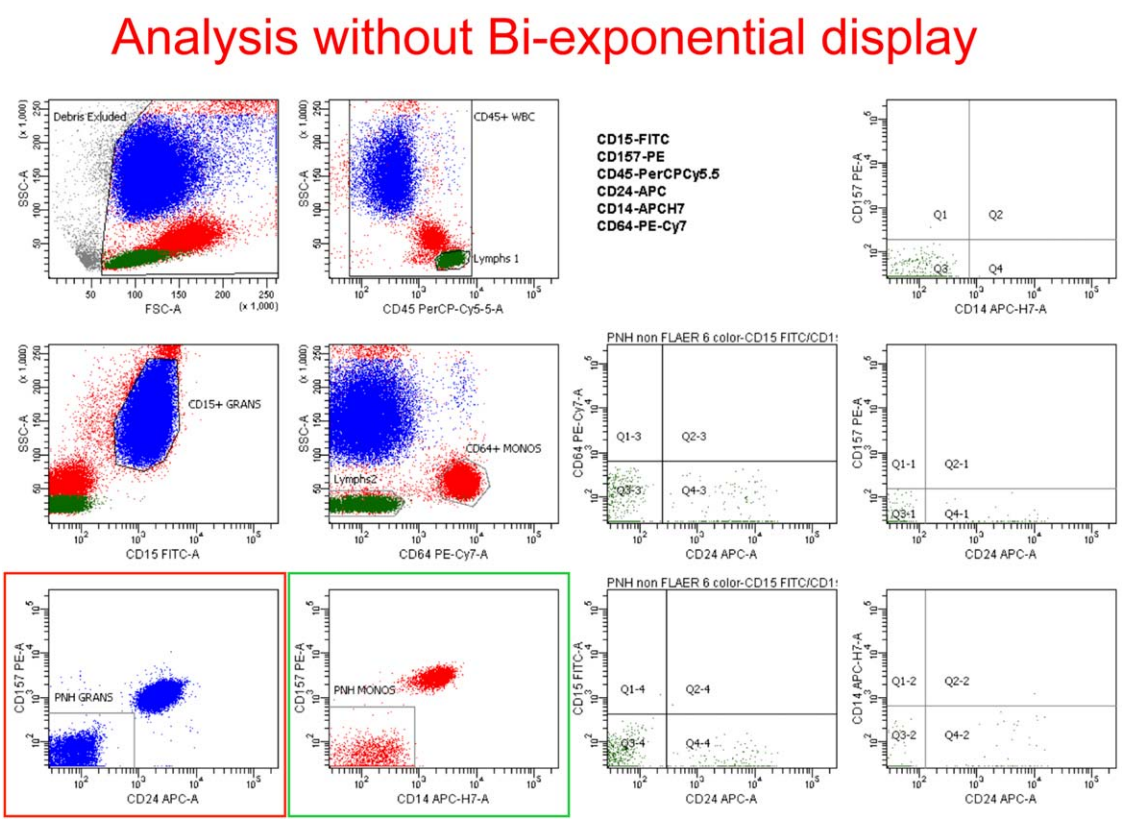

FIG. A2. Same data file as figure 1, without bi-exponential display. PNH neutrophils and PNH monocyte populations are not fully visible/on-scale indicating that the PMT settings are too low for this assay. With respect to the lymphocytes, that serve as internal control subsets to monitor instrument voltage and compensation, and assess antibody performance for PNH assays, very few are visible at such settings. [Color figure can be viewed at wileyonlinelibrary.com]

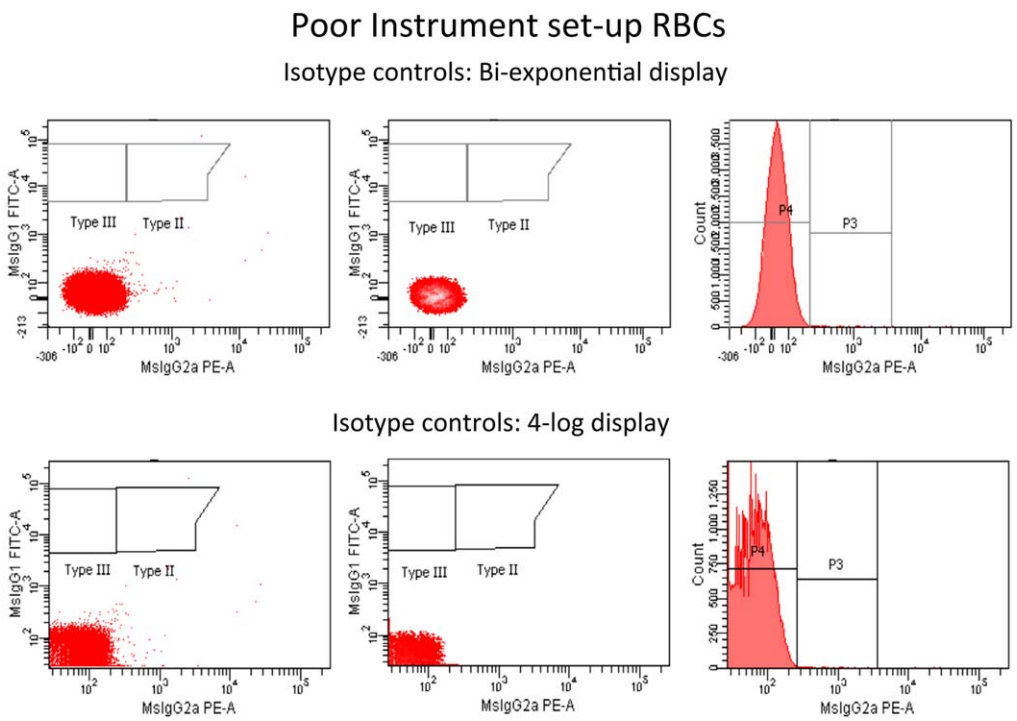

FIG. A3. Unstained RBCs on FACSCanto set up using manufacturer's generic PMT settings. PMT settings are too low to see unstained (or in this specific case, isotype-stained) RBCs when viewed in 4- or 5-decade log scale (bottom row). Only in bi-exponential scale can all the RBCs be visualized on scale (top row, right). [Color figure can be viewed at wileyonlinelibrary.com]

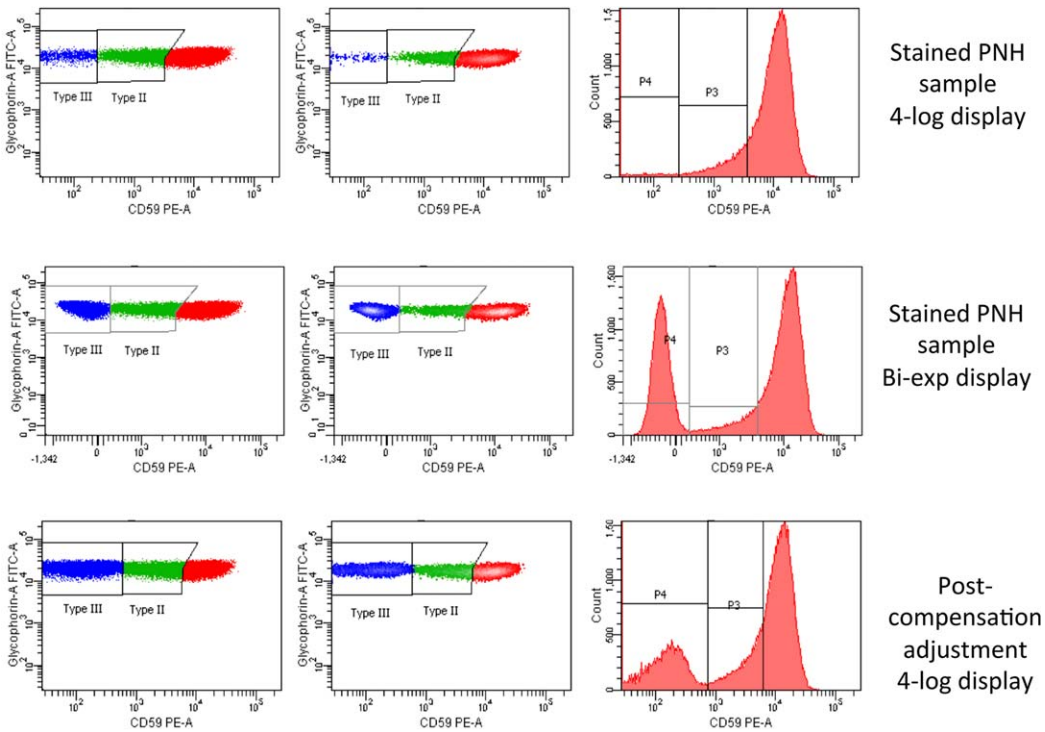

FIG. A4. PNH sample stained with CD235aFITC (clone KC16) and CD59PE (clone MEM-43) with a Type III RBC content of 33.3% based on statistics from the single parameter histogram (top right). However, the Type III cells are not visible. Only in bi-exponential display (middle row right), can one gain any confidence that this sample indeed contained 33.3% PNH Type III RBCs. Not all of the problems visualizing the data from this sample were due to the obviously too low PMT setting; removing some of the over-compensation between PMT1 and PMT2 allowed the majority of the Type III RBCs to be visualized in 4-decade format (lower right). [Color figure can be viewed at wileyonlinelibrary.com]

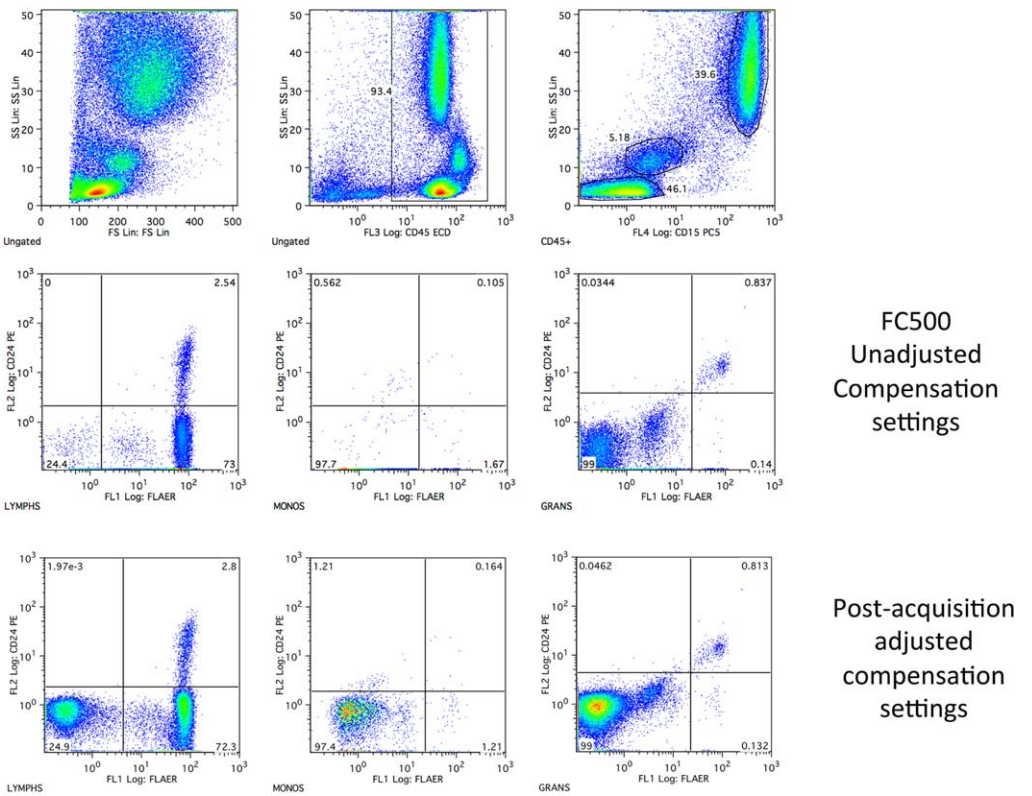

FIG. A5. PB sample stained with FLAER (Cedarlane), CD24PE (clone ALB9), CD45ECD (clone J33) and CD15PC5 (clone 80H5) and analysis performed on Beckman Couter FC500. The use of suboptimal PMT voltages coupled with over-compensation resulting from the use of saturating volumes of CD45 conjugates to set the compensation matrix has led to PNH neutrophils, PNH monocytes and PNH lymphocytes being crushed on FL1 (FLAER) and FL2 (CD24-PE) axes (Middle row). Even after removing the over-compensation of CD45-ECD from the FLAER and CD24-PE channels, the FL1 PMT voltage is too low to visualize all cells of interest fully onscale (bottom row). [Color figure can be viewed at wileyonlinelibrary.com]